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Nomenclature of Amino-acids

The following rules have been agreed upon between the Editors of the *Journal of the Chemical Society* and of the *Biochemical Journal*, after consultation with their American colleagues. The examples are given solely to illustrate the application of the rules; in other respects (e.g. the method of enumeration of carbon atoms) names will naturally follow the customary usages of the journal in which they are to appear.

RULE 1. In cases where no possibility of misunderstanding can arise regarding which isomer of the amino-acid is under discussion, the amino-acid may be named without prefix or symbol to denote the direction of the rotation.

RULE 2. In cases where distinction between the stereoisomers is necessary, or considered desirable for the sake of clarity, the configurational family to which the *alpha carbon atom* belongs should be designated by a prefixed *small capital letter*. An additional sign to denote the direction of the rotation is not necessary.

Examples: L-leucine, D-valine.

The optically inactive mixture or racemic compound should, except where Rule 1 applies, be designated with the prefix DL.

Example: DL-leucine.

RULE 3. The configuration of carbohydrates and other compounds which can be configurationally related to glyceraldehyde is designated by a prefixed D or L. Where confusion is possible between the use of the small capital letter prefix for the configuration of the *alpha carbon atom* in amino-acid nomenclature and for that of the highest numbered asymmetric carbon atom in carbohydrate nomenclature, a subscript is added to the small capital letter prefix. Where the prefix is used in the amino-acid sense, the subscript '*s*' is added; where the prefix is used in the carbohydrate sense, the subscript '*g*' is added. These subscripts refer, respectively, to serine, the fundamental substance to which amino-acids that bear structural resemblance to the carbohydrates can be formally related, and to glyceraldehyde, the fundamental substance to which the configuration of the carbohydrates is formally related.

Examples: L_s-threonine, for which the synonym in carbohydrate nomenclature is 2-amino-2:4-dideoxy-D_g-threonic acid; D_s-threonine, for which the synonym is 2-amino-2:4-dideoxy-L_g-threonic acid; L_s-*allo*threonine, for which the synonym is 2-amino-2:4-dideoxy-L_g-erythronic acid; D_s-*allo*threonine, for which the synonym is 2-amino-2:4-dideoxy-D_g-erythronic acid.

Papers that deal with both carbohydrates and amino-acids and which make use of the prefixes should employ subscripts at least for the names of the amino-acids. The acids derived from amino sugars should be named in conformity with carbohydrate nomenclature but with the use of the subscript: e.g. D_g-glucosaminic acid for 2-amino-2-deoxy-D_g-gluconic acid.

RULE 4. If the configurational relationship of the *alpha carbon atom* has not been definitely established, the actual direction of the rotation in a specified solvent, preferably of the free amino-acid in water, is designated by the prefixes *dextro* or *laevo* or, alternatively, by a plus or a minus sign enclosed in parenthesis.

Examples: *dextro*hydroxytryptophan, (+)-hydroxytryptophan.

RULE 5. The prefix *meso* should be used for the *meso* isomers of amino-acids and their derivatives that are optically inactive because of internal compensation.

Example: *meso*lanthionine, *meso*cystine.

RULE 6. Where the amino-acid has two asymmetric centres, but where internal compensation is impossible, the form which occurs in proteins should be called the L-amino-acid, and the form which has opposite configuration on both asymmetric carbon atoms should be designated the D-amino-acid. The pair having opposite configurations on one asymmetric centre only should be designated D-*allo* and L-*allo*, D and L referring as usual to the *alpha carbon atom*.

Examples: D-*allo*threonine, L-*allo*hydroxyproline.

RULE 7. Derivatives and salts of amino-acids and peptides should, where necessary, be named with the use of D or L to denote the configurational family of the *alpha carbon atom* or *atoms*, the customary rules being otherwise observed.

Examples: acetyl-L-tryptophan, L-histidine monohydrochloride monohydrate, copper L-aspartate, D-leucyl-D-valine.

RULE 8. Where a trivial name is applied to a compound that contains two alpha amino carboxylic acid groupings attached to dissimilar carbon chains, the configurational designation is that of the *alpha carbon atom* of the *smaller of the two chains*.

Examples: D-cystathionine for *S*-(D-β-amino-β-carboxyethyl)-D-homocysteine, L-*allo*cystathionine for *S*-(L-β-amino-β-carboxyethyl)-D-homocysteine.

It is emphasized that the introduction of new trivial names should be avoided.

1948

The Liberation of Glucuronic Acid from Conjugated Glucuronides by β-Glucuronidase

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(*Received* 18 *April* 1947)

Aqueous extracts of many animal tissues have been found to contain an enzyme, 'β-glucuronidase', which catalyzes the hydrolysis of β-glucuronides. It has been suggested (Oshima, 1934; Fishman, 1940; Fishman & Fishman, 1944) that the production of conjugated glucuronides *in vivo* is due to this enzyme. The activity of β-glucuronidase preparations has usually been determined by measuring the reducing material formed on incubation with a glucuronide (Masamune, 1934; Oshima, 1934, 1936; Fishman, 1939, 1940; Fishman & Fishman, 1944; Graham, 1946; Mills, 1946). The use of such methods rests on the assumption that the hydrolytic action of the enzyme is confined to rupture of the glucosidic link. Evidence for this is indirect, and is confined to experiments by Masamune (1934), who determined colorimetrically the free menthol in enzyme hydrolysates of its glucuronide and found it to correspond to the reducing material produced when the latter was calculated as glucuronic acid. It was considered that this point required further investigation in view of the discovery by Lipschitz & Bueding (1939) that synthesis of glucuronides by liver slices is stimulated, not by glucuronic acid, but by certain C_3 compounds, such as pyruvic acid.

In the first series of experiments described below, glucuronic acid liberated from menthylglucuronide by β-glucuronidase was separated from unchanged substrate and determined by means of the colour reaction with naphthoresorcinol. The results agreed with estimates of free glucuronic acid obtained by reduction of ferricyanide, suggesting that the hydrolysis proceeds according to the generally accepted view. Since, however, the colour reaction with naphthoresorcinol is not specific for glucuronic acid (Mandel & Neuberg, 1908; Dische, 1947), a second series of experiments was carried out in which the method of Lohmar, Dimler, Moore & Link (1942) was used to isolate and identify the uronic acid.

EXPERIMENTAL

General methods

Biosynthetic *l*-menthyl-D-glucuronide (Williams, 1938) was used as substrate in all the experiments. β-Glucuronidase was prepared from beef spleen according to the directions given by Graham (1946). Unless stated to the contrary, the enzyme was highly purified (stage *G* in Graham's method), and had a negligible 'reducing blank', even after incubation. Reducing material in hydrolysates of menthylglucuronide was estimated by the procedure previously described (Levvy, 1946). The determination of free glucuronic acid and of menthylglucuronide by means of the colour reaction with naphthoresorcinol was done by the method of Hanson, Mills & Williams (1944). In agreement with these authors, D-glucurone and menthylglucuronide were found to be equivalent in colour production.

Free glucuronic acid and reducing material in hydrolysates

Procedure. To 0·5 ml. enzyme in a 10 ml. centrifuge tube were added 1·0 ml. 0·1 M-acetate buffer, pH 5, and 0·5 ml. substrate solution (20 mg. menthylglucuronide/ml., adjusted to pH 5). The tube was stoppered and placed in a bath at 37°. At the end of a predetermined period, protein was precipitated with $CuWO_4$ and $BaCO_3$ (Levvy, 1946), using 1·5 ml. of each of the two solutions specified. After centrifuging, 3 ml. of the supernatant was removed and diluted to 25 ml. From this solution, 1 ml. samples were taken for the determination of reducing material, and 0·4 ml. samples for the colorimetric estimation of total glucuronic acid (after prior dilution to 2 ml.). Free glucuronic acid was determined in 1 ml. samples after acidification with 1 ml. 0·02 N-H_2SO_4 and extraction with peroxide-free ether for 1 hr. (see below). Before developing the colour, the aqueous layer was freed from ether by placing the extraction tube in hot water and blowing air over the surface of the liquid.

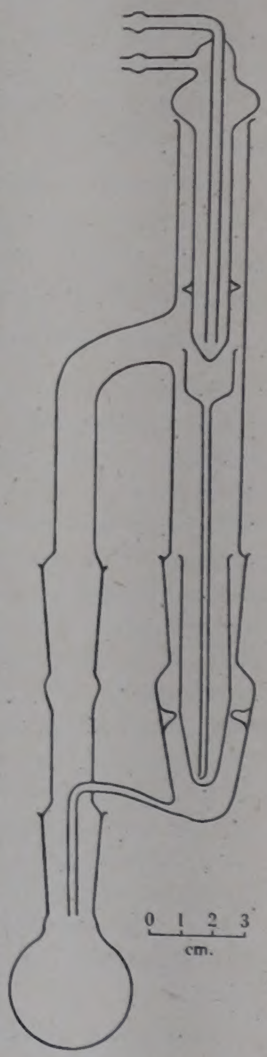

Fig. 1. Micro-liquid-liquid extraction apparatus.

The separation of free and combined glucuronic acid. The removal of unchanged menthylglucuronide from free glucuronic acid by ether extraction was carried out in the specially designed micro-liquid-liquid extractor shown in Fig. 1. The aqueous layer was left at the end of the extraction in a convenient form for further manipulation, and since the extraction chamber could readily be kept cool a very rapid stream of ether could be maintained through the solution. No difficulties were encountered in the manufacture of the apparatus in spite of the double joint. When the glass tube for return of ether to the flask was softened by heating a good fit at both joints was readily obtained. The course of extraction of known amounts of menthylglucuronide under conditions resembling those in the experiments described above is shown in Fig. 2. Free glucuronic acid was not extracted under these conditions.

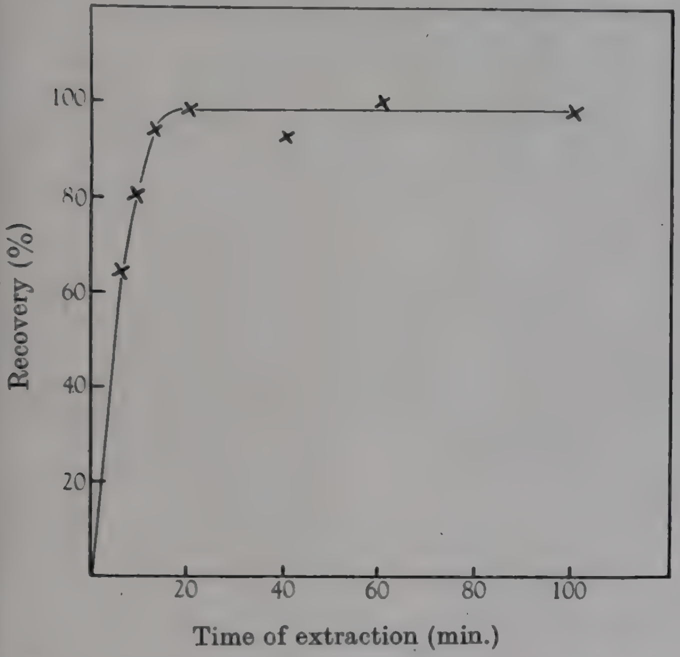

Fig. 2. Extraction of menthylglucuronide with ether.

Results. Fig. 3 shows the course of hydrolysis of menthylglucuronide followed colorimetrically and by ferricyanide reduction. Similar results were obtained with another enzyme preparation. It should be pointed out that the average recovery of glucuronic acid, free and combined, corresponded to only 85 % of the total originally added as menthylglucuronide, suggesting retention by the protein precipitate. There would appear, however, to be no doubt that the reducing material in the filtrate was quantitatively accounted for as glucuronic acid.

The isolation of glucuronic acid as the dibenziminazole derivative of D*-glucosaccharic acid*

The hydrolysis. Menthylglucuronide (700 mg.) was dissolved by warming in 10 ml. 0·4N-acetate buffer. When cool, the pH was adjusted to 5 (glass electrode). To the solution was added 10 ml. of an enzyme preparation containing a total of 500 'glucuronidase units' (Graham, 1946). The mixture was kept at 37° for 24 hr., and then dialyzed through Visking sausage casing against 80 ml. water in the refrigerator for a further 24 hr. After acidifying with 2 ml. 11N-H_2SO_4, the dialysate was extracted with ten 60 ml. portions of peroxide-free ether in a separating funnel to remove menthol and unreacted substrate. The aqueous layer was shaken vigorously with 4 g. powdered $BaCO_3$, added in small portions, till the solution was free from sulphate ion, and filtered. The pH of the filtrate, originally about 7·5, was brought to 7 with HCl, and water was removed *in vacuo*. The residue was dissolved in 10 ml. water and, after removal of 0·2 ml. for determinations of reducing material, evaporated once more to dryness. The use of ethanol to remove last traces of water was considered inadvisable in view of the risk of esterification. From its reducing power, the total residue appeared to contain 148 mg. glucuronic acid. When correction is made for glucuronic acid left in the dialysis sac, this corresponds to 49 % hydrolysis of the substrate. Control experiments showed that no glucuronic acid was liberated in the absence of enzyme under the conditions of the experiment, and that menthylglucuronide was completely removed from aqueous solution by the extraction process.

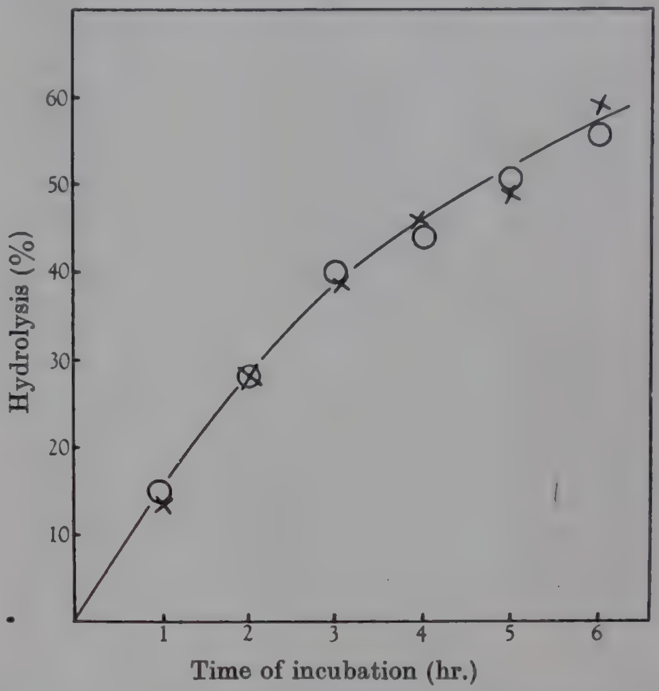

Fig. 3. The enzymatic hydrolysis of menthylglucuronide determined colorimetrically and by reduction of ferricyanide. ×, reduction; o, colour reaction.

Isolation of the dibenziminazole. The residue from the hydrolysis was taken up in 16 ml. water containing 800 mg. barium benzoate. After cooling to 0°, 0·15 ml. bromine was added. The oxidation mixture was kept in the dark at room temperature for 1 day and at 25° for a second day. Excess bromine, ions and benzoic acid were removed (Hudson & Isbell, 1929) and the saccharic acid coupled with o-phenylenediamine as described by

Lohmar *et al.* (1942); 60 mg. of the dibenziminazole, m.p. 234–235° (corr.), was obtained (22 % of the theoretical in terms of glucuronic acid in the hydrolysis residue).

The hydrolysis was repeated using only 300 units of enzyme, but continuing the incubation for 48 hr. From its reducing power, the residue in this case contained 195 mg. of glucuronic acid (corresponding to 65 % hydrolysis) and yielded 105 mg. of the iminazole (30 % of theoretical), m.p. 235–236° (corr.). This was combined with the product of the first experiment and put through one stage of purification, giving a final yield of 130 mg. The dihydrochloride and dipicrate were prepared and, as shown by the data in Table 1, the identity of the iminazole with an authentic specimen prepared from D-glucosaccharic acid was proved. Since both the hydrochloride and picrate were found to be hydrated, details of their preparation and figures obtained on analysis are given below.

The hydrochloride. To prepare the hydrochloride, the free base was dissolved in warm N-HCl (0·1 g./ml.). The crystals which separated in the refrigerator were washed with a few drops of ice-cold water and a large volume of acetone. If necessary the hydrochloride was recrystallized from a very small volume of water. After drying *in vacuo* over $CaCl_2$ at room temperature it was obtained as the tetrahydrate. An authentic specimen gave: C, 43·42; H, 5·71 % (calc. for $C_{18}H_{20}O_4N_4Cl_2 . 4H_2O$: C, 43·29; H, 5·62 %). After drying for 10 hr. *in vacuo* at 110° over P_2O_5, the loss in weight was 14·63 % (calc. for the tetrahydrate, 14·42 %). The dried material gave: C, 50·05; H, 5·07 % (calc. for $C_{18}H_{20}O_4N_4Cl_2$: C, 50·58; H, 4·68 %). Lohmar *et al.* give the m.p. as 257–258° (uncorr.).

The picrate. To prepare the picrate, the hydrochloride was dissolved in hot water and excess picric acid was added as a hot, saturated aqueous solution. The liquid was allowed to cool slowly. In this way, separation as an oil was avoided. The crystals were washed with water and, if necessary, recrystallized from a large volume of water. After drying *in vacuo* over $CaCl_2$ at room temperature, this compound was obtained as the trihydrate. An authentic specimen gave: N, 16·35 % (calc. for $C_{30}H_{24}O_{18}N_{10} . 3H_2O$: N, 16·17 %). After drying for 10 hr. *in vacuo* at 110° over P_2O_5, the loss in weight was 5·79 % (calc. for the trihydrate, 6·23 %). The dried material gave: C, 44·47; H, 3·06 % (calc. for $C_{30}H_{24}O_{18}N_{10}$: C, 44·33; H, 2·96 %). Lohmar *et al.* give the m.p. as 211° (uncorr.).

The reducing material in crude enzyme preparations

In the experiments previously described, highly purified β-glucuronidase, containing little if any reducing material, was employed. It was considered of interest to discover whether the reducing material in crude enzyme preparations could be accounted for entirely or in part as glucuronic acid. Two preparations of β-glucuronidase at stage *A* in Graham's (1946) procedure were taken for this purpose. After removal of protein with $CuWO_4$ and $BaCO_3$, both were found to contain very large amounts of reducing substances (equivalent to 0·75 and 0·48 mg. glucuronic acid/ml. respectively) before incubation. After incubation at 37° for 3 hr. in acetate buffer at pH 5 in absence of added substrate, the reducing material in the latter preparation was equivalent to 0·64 mg. glucuronic acid/ml. In no case did the protein-free filtrate give any colour in the reaction with naphthoresorcinol.

Table 1. *Properties of benziminazole derivatives from glucuronic acid*

	Substance isolated	Authentic specimen	Mixture
Dibenziminazole*	m.p. 243°	m.p. 240–242°	m.p. 240°
Dihydrochloride*	m.p. 265°; $[\alpha]_D^{18^\circ} = +52{\cdot}3$† (S.E. 0·64°)	m.p. 267°; $[\alpha]_D^{16^\circ} = +52{\cdot}3$‡ (S.E. 0·62°)	m.p. 265–266°
Dipicrate*	Change of form 145°; m.p. 211°; C, 41·83 %; H, 3·20 % (calc. for $C_{30}H_{24}O_{18}N_{10} . 3H_2O$: C, 41·57 %; H, 3·47 %)	Change of form 145°; m.p. 211°	Change of form 140°; m.p. 211°

* All melting-points corrected. Decomposition before or at melting-point in every case.
† 10·123 mg. tetrahydrate in 0·5 ml. water.
‡ 20·253 mg. tetrahydrate in 1·0 ml. water.

DISCUSSION

From the results of the experiments described above, there would appear to be no doubt that the hydrolytic action of the enzyme known as β-glucuronidase is confined to the rupture of the glucosidic link in conjugated glucuronides. There is thus a distinction to be drawn between 'β-glucuronidase' and the enzyme system which synthesized glucuronides in the experiments of Lipschitz & Bueding (1939), since the latter appeared to build up the hexuronic acid fraction of the molecule from C_3 compounds. In the light of this, it is difficult to accept the postulate of Oshima (1934) and Fishman (1940) that 'β-glucuronidase' is responsible for the production of conjugated glucuronides in the body by a reversal of the hydrolytic action seen *in vitro*. Fishman put forward in support of this view his observation that prior administration of glucuronidogenic compounds to animals increased the glucuronidase content of certain tissues (Fishman, 1940; Fishman & Fishman, 1944). As an explanation of his experimental data, it would seem at least equally plausible to suppose that 'β-glucuronidase' acts purely as a hydrolytic enzyme in the body, and that increase in the amount of enzyme in an organ occurs in response to the presence of excess glucuronide formed from the aglycone administered. The work of Mills (1946), who found that splenectomized rabbits fed with menthol conjugated larger fractions with glucuronic acid than normal, could be more readily explained by the latter theory.

Since the reducing material formed from menthylglucuronide can be accounted for as glucuronic acid, it appears that estimates of β-glucuronidase activity based on the former are sound. Considerable practical difficulties may, however, be encountered in assaying the enzyme in this way if the preparation is at all impure. The preparation may initially contain large amounts of reducing substances, and these may increase when the enzyme is incubated in absence of substrate (Graham, 1946). Heat inactivation of the enzyme prevents further production of reducing material. Graham therefore recommended that controls in determining glucuronidase activity should consist of enzyme incubated for the same length of time as the reaction mixture, instead of heat-inactivated enzyme as used by Fishman (1939). Graham's procedure would not, however, be correct if the reducing material in the controls were derived from a glucuronide, since in the experimental tubes the latter would merely add to the excess substrate. Enzyme preparations with very high reducing powers were found to give completely negative reactions with naphthoresorcinol, indicating the absence of glucuronic acid, free or in combination as a glucuronide. It appears safe to assume that production of the unknown reducing substances will proceed at the same rate in experimental and control tubes, independently of the enzyme's action, and that Graham's procedure is therefore justifiable.

SUMMARY

1. The reducing material formed when menthylglucuronide was incubated with freshly purified beef spleen β-glucuronidase was accounted for by free glucuronic acid, determined by the colour reaction with naphthoresorcinol.

2. The glucuronic acid in enzyme hydrolysates of menthylglucuronide was isolated and characterized as the dibenziminazole derivative of D-glucosaccharic acid.

3. Crude preparations of β-glucuronidase, containing large amounts of reducing material, did not give the colour reaction for glucuronic acid and conjugated glucuronides.

4. The theoretical implications of these findings are discussed.

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Preliminary Studies on the Carbohydrate-rich Fractions of Ox Serum

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(*Received* 7 *March* 1947)

The glycoproteins of blood have formed the subject of numerous researches. Their existence having been once established (Bywaters, 1909; Biery & Ranc, 1914; Rimington, 1929), investigators have attempted either their isolation or the elucidation of the nature of the carbohydrate-containing complex. Attempts at isolation (Meyer, 1938; Sørensen, 1938; Hewitt, 1936, 1937; Rimington, 1931, 1940) have not led to a product which has been characterized as pure by the modern physico-chemical criteria available (e.g. electrophoresis, ultracentrifugation). McMeekin (1940) has recently announced the isolation of a crystalline carbohydrate-containing albumin fraction which was also electrophoretically homogeneous, but these two criteria alone are insufficient evidence of purity, and we decided therefore to apply to this substance further tests including the rigorous one of constant solubility under a given set of conditions. Unfortunately the procedure of isolation recorded is extremely laborious, and although we have seen crystals answering to the description given by McMeekin we have not been able to obtain workable quantities of the substance.

The constitution of the polysaccharide moiety of the blood glycoproteins has not as yet been unequivocally settled. Are we dealing with a galacto-mannose-glucosamine, a gluco-galactose-glucosamine or even with several different polyosides? Penet (1940), Dumazert & Memmi (1943) and Dumazert, Lévy & Marszak (1944) favour the latter view. Dumazert & Lévy (1943) isolated and attempted to identify a carbohydrate fraction from the plasma which is not present in the free state but in an easily detachable form. This fraction, which they call 'a loosely combined carbohydrate', would be the same in their view as the 'sucre virtuel' of Lépine (1921). It contains no glucosamine but only glucose and might, they suggest, be composed of three molecules of glucose united to one of phosphoric acid. Such a substance would clearly be of considerable physiological interest.

It is with two main problems in mind, purification of starting material and elucidation of chemical constitution, that we have undertaken the more extended study of a glycoprotein, a seromucoid fraction previously obtained by one of us (Rimington, 1940) from serum. In particular we have attempted (1) to explore the solubility curve of our mucoid in the presence of a precipitant, and (2) to cleave the carbohydrate-protein linkage by methods more gentle than acid or alkaline hydrolysis and to investigate the polyosides so obtained.

MATERIAL AND METHODS

Starting material

This was a seromucoid fraction obtained after removal of heat-coagulable protein from ox blood and precipitation by 50% (v/v) ethanol at pH 4. It was thus equivalent to seromucoid fraction *e* described by Rimington (1940, p. 933).

Analytical methods

Total hexose. Both the orcinol method as described by one of us (Rimington, 1940) and the carbazole method of Gurin & Hood (1939) have been employed, the latter affording somewhat more consistent figures. A mixture of equal parts of mannose and glucose served as reference standard except when examining one fraction by the carbazole method when a glucose standard was used (see discussion). Comparison was made in a Pulfrich step photometer, the orcinol colours always being read through both filters S 47 and S 50, whilst for carbazole colours, filter S 50 was employed. Results obtained by the carbazole method were higher by a factor of about 1·4 than those obtained with orcinol but, allowing for this factor, agreement was very good. (Gurin & Hood (1939) have already observed that carbazole reacts to some extent with amino-acids.) In any one series of experiments the same method was used throughout.

Glucosamine. The acetylacetone and Ehrlich reagent technique has been employed under the conditions already described (Rimington, 1940) using filter S 53.

Nitrogen was determined by the micro-Kjeldahl method using selenium oxide catalyst.

Precipitation curves were based upon the specific property phase rule relationship enunciated by Falconer & Taylor (1946). Since the hexose/nitrogen (H/N) ratio is characteristic for a glycoprotein, we have constructed curves expressing (*a*) soluble nitrogen and (*b*) soluble hexose in relation to concentration of precipitant, and (*c*) soluble hexose as a function of soluble nitrogen.

All equilibrations were carried out at 37° when using Na_2SO_4 and at laboratory temperature when using other salts or ethanol. The different weights (or volumes) of precipitants were introduced into Emich micro-filtration vessels and then a constant volume of the seromucoid solution. The vessels were stoppered, and after the required conditions of time and temperature had been established, filtration was effected through the sintered glass disk by applying a positive pressure of nitrogen.

RESULTS

Precipitation curves and fractionation by Na_2SO_4

Preparation of the seromucoid solution

For all experiments, a solution of the starting material was made as follows. To a weighed quantity of mucoid, distilled water was added to yield a 0·2–0·3% (w/v) solution and the mixture dialyzed against distilled water for 2–3 days. It was then filtered through no. 1 Whatman paper, the latter washed with washings of the dialyzing bag and the volume adjusted to 1 g./l., assuming complete solubility of the protein. This technique afforded more complete solution than simple addition of distilled water (Table 1, compare solution 1 with 2, 3 and 4). The solutions which had a pH between 4 and 5 were used without neutralization and afforded very constant values for the ratio hexose/nitrogen (see Table 1).

Table 1. *Analytical data from different seromucoid solutions prepared as described in text from the same raw material*

Solution	Nitrogen (μg./ml.)	Hexoses by orcinol method using glucose-mannose standard (μg./ml.)	$\frac{\text{Hexose}}{\text{Nitrogen}}$
1	155	148	1·04
2	200	196	1·02
3	200	190	1·05
4	200	194	1·03

Choice of precipitant

The characteristics of several precipitants were investigated with the following results.

Ethanol. Although in presence of sodium acetate this caused complete precipitation of seromucoid, the concentration zone of precipitation was too restricted for useful application to the purpose in hand; moreover the volatility of ethanol and the unavoidable dilution of the starting material introduced troublesome sources of error.

Magnesium sulphate and alkaline phosphates were found not to precipitate seromucoid.

Ammonium sulphate. Although capable of precipitating seromucid, the use of this salt was abandoned for analytical reasons, since no agent could be found which would remove the protein completely from solution and thus enable the micro-Kjeldahl method to be employed without preliminary dialysis. Seromucoid is neither heat-coagulable nor is it completely precipitable by phosphotungstic acid, hot tungstic acid or trichloroacetic acid.

Sodium sulphate. Both the anhydrous salt and $Na_2SO_4 . 12H_2O$ precipitated at 37° the major part of the seromucoid and below are given the results obtained with the anhydrous salt, all operations having been conducted in a hot room.

Solubility curves in presence of sodium sulphate

Quantities of anhydrous sodium sulphate varying from 1 to 2·75 g. were weighed and then to each were added exactly 5 ml. of a seromucoid solution prepared as described above. Solution was effected as rapidly as possible by warming the vessel and contents in a water bath at 37°, but with mixtures containing a high concentration of salt the solution

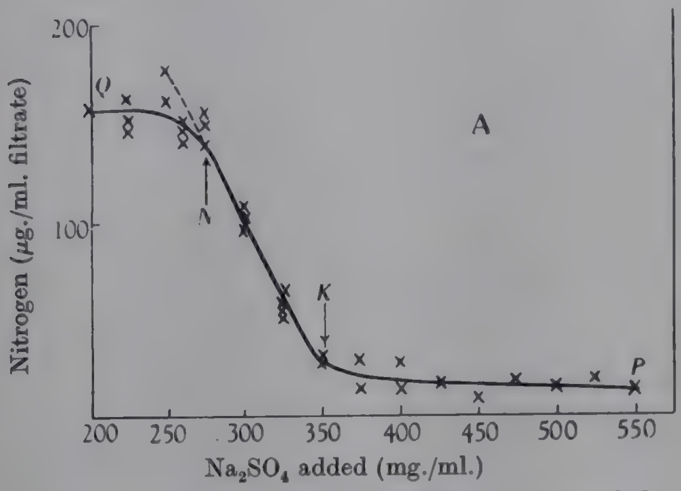
Table 2. *Salting out of seromucoid by sodium sulphate; data for solubility curves*

(Two different starting solutions were used (A and B) and the data were obtained in four separate series of experiments performed at different dates indicated by the numbers 1, 2, 3 and 4 respectively.)

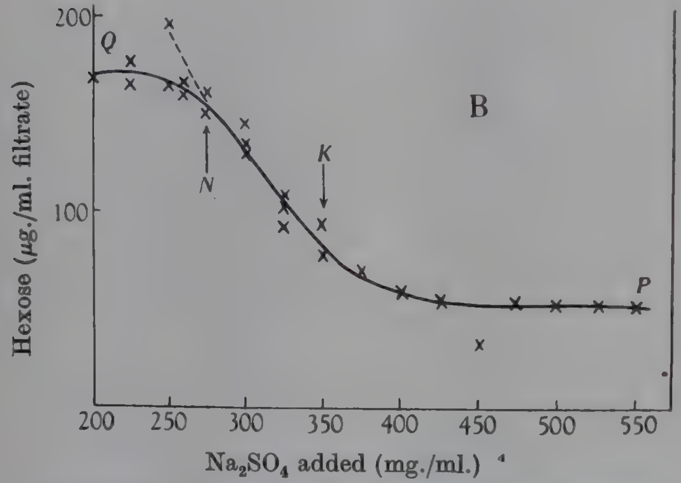
Na_2SO_4 added (mg./ml.)	Correction factor $k=(1+x)/b$	Seromucoid solution	Experimental series	Nitrogen in filtrate		Hexoses in filtrate by orcinol method	
				Found (μg.)	Corrected (μg.)	Found (μg.)	Corrected (μg.)
200	1·042	A	1	153	159	160	167
225	1·049	A	1	155	162	168	176
		A	2	144	151	155	163
		A	4	140	147	155	163
250*	1·054	A	1	146	154	155	163
		A	2	152	160	155	163
		B	3	168	177	175	184
260	1·059	A	1	138	146	155	164
		A	2	140	148	150	159
		A	4	132	140	155	164
275	1·062	A	1	132	140	150	159
		A	2	146	155	140	149
		A	4	140	149	150	159
300	1·068	A	1	98	105	120	128
		A	2	96	102	125	133
		A	4	96	102	135	144
		B	3	90	96	120	128
325	1·075	A	1	48	52	85	91
		A	2	50	54	95	102
		A	4	54	58	100	107
		B	3	60	64	102	109
350	1·080	A	1	32	34	82	88
		B	3	30	32	87	94
375	1·087	A	1	16	17	65	70
		B	3	28	30	65	70
400	1·095	A	1	14	15	57	62
		B	3	26	28	55	60
425	1·101	B	3	16	18	50	55
450†	1·104	A	1	9	10	30	33
475	1·104	B	3	16	18	50	55
500	1·104	B	3	14	15	50	55
525	1·104	B	3	17	19	50	55
550	1·104	B	3	12	13	50	55

* Ppt. first visible. † Saturation.

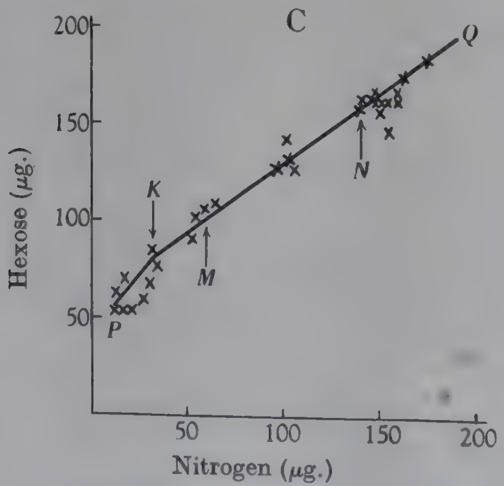
was slow and the vessels required periodic shaking for the first few hours. After 24 hr. a precipitate of protein was present in the series which contained a concentration of Na_2SO_4 greater than 250 mg./ml. Filtration was carried out at 37° under a positive pressure of N_2 and samples of 1 ml. of filtrate taken for duplicate nitrogen and hexose determinations. Hexose was frequently determined in triplicate.



A. Relation between soluble nitrogen and salt added.



B. Relation between soluble hexoses and salt added.



C. Relation between hexoses and nitrogen.

Fig. 1. Salting out of seromucoid by sodium sulphate at 37° (for explanation of lettering see text).

In order that all results should refer to 1 ml. of the original solution of glycoprotein, the experimentally determined values were corrected by a factor k derived from the formula $k=(a+x)/b$, where a=sp.gr. of the original solution of seromucoid, x=weight of Na_2SO_4 added to 1 ml. of this solution and b=sp.gr. of the final solution. In practice, since the solution of seromucoid is very dilute, we have calculated values of k for solutions of Na_2SO_4 of various concentrations in distilled water from the expression $k=(1+x)/b$.

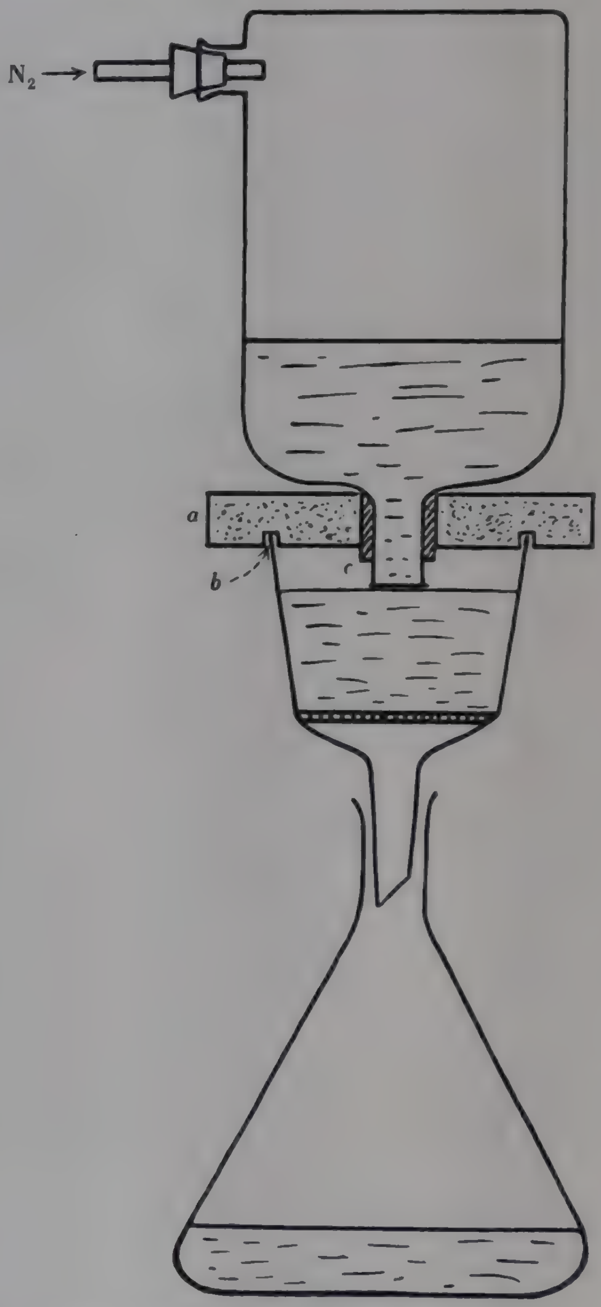


Fig. 2. Filtering device used for fractionation of large quantities of seromucoid by sodium sulphate at 37°. a, wooden block; b, wax-lined groove; c, rubber sleeve.

The results of solubility curve determinations carried out in four series of experiments on different solutions of seromucoid exhibit a satisfactory concordance (Table 2 and Fig. 1). They will be discussed in detail below.

Fractionation of seromucoid by Na_2SO_4

On the basis of information obtained from the solubility curves, a fractionation of seromucoid was carried out as follows: to 450 ml. of the seromucoid solution were added 123·75 g. of anhydrous Na_2SO_4 (0·275 g./ml.) and the mixture kept at 37° for 24 hr., agitation being frequent during the first few hours. Filtration through a large sintered glass filter (porosity G 4) without loss from evaporation was accomplished by the use of a device (Fig. 2) sealing hermetically the solution-container to the body of the filtering funnel. The precipitate was dissolved from the funnel with successive quantities of water and dialyzed, yielding fraction Na I. After further addition of 22·5 g. Na_2SO_4 (0·05 g./ml.) to the filtrate, a further precipitate was similarly removed affording fraction Na II. The final fraction Na III was obtained after a further 78·75 g. Na_2SO_4 (0·175 g./ml.) had been added to the filtrate to achieve full saturation with the salt.

Nitrogen, hexose and hexosamine determinations were made on fractions Na I and Na III but fraction Na II was unfortunately lost before analysis. The analytical results are presented in Table 3 and are further discussed below.

Cleavage of seromucoid by phenol

Seromucoid in the form of a powder was added to 92 % (w/v) phenol according to a procedure used by Morgan & Partridge (1941) and dialyzed. The hexose/total N ratio was determined in the water-soluble and water-insoluble fractions afforded by the dialysis.

Fractionation of phenol solutions by ethanol. The solutions of the seromucoid in phenol were either dialyzed directly against distilled water, or were first precipitated by addition of ethanol, the insoluble material being subsequently suspended in water and dialyzed. The latter procedure was more satisfactory, the H/N ratio of the water-soluble fraction after dialysis being higher (Table 4). Moreover this carbohydrate-rich fraction is precipitated completely from the phenolic solution by the addition of 1 vol. of ethanol, further additions of ethanol only bringing down products with a H/N ratio of 0·24–0·70 as compared with 3–4.

Study of the fraction insoluble in water after dialysis of the ethanol-phenol precipitate. A portion of the precipitate was dissolved in a little 0·05N-NaOH, the solution neutralized and dialyzed. Analysis of the solution indicated a fall in the ratio of hexose to nitrogen ($H/N = 0·77$) relative to that of the original (or 'standard') preparation of seromucoid ($H/N = 1·04$). The remainder of the precipitate was washed with water, dried *in vacuo*, powdered and re-extracted by 92 % (w/v) phenol. Only a small quantity dissolved and this was worked up as on the former occasion by addition of ethanol, dialysis, etc. The residual water-insoluble material was dissolved in NaOH, neutralized, dialyzed and analyzed as above, but no further decrease of the H/N ratio was apparent. It does not seem possible, therefore, to separate by this means the residual polyoside from

Table 3. *Composition of fractions obtained from seromucoid by fractionation with either sodium sulphate or phenol and chloroform*

Fraction	Nitrogen (%)	Hexoses by carbazole method: (*a*) glucose-mannose standard, (*b*) glucose standard (%)	Glucosamine (%)	Hexose / Nitrogen	Glucosamine / Hexose
Seromucoid	13·0	17·8 (*a*)	8·0	1·44*	0·45
Na I	11·8	11·6 (*a*)	6·7	0·94	0·58
Na III	12·5	15·2 (*a*)	8·7	1·15	0·57
C I	3·9	66·0 (*b*)	10·0	17·0	0·15
C II	11·4	12·4 (*a*)	7·4	1·08	0·60

* The difference from the figure given in Table 1 is due to the use of the carbazole in place of the orcinol method (see text).

Table 4. *Fractions obtained from a solution of seromucoid in phenol by dialyzing directly or by dialyzing after a preliminary ethanol precipitation*

Origin of fraction	Dry wt. (mg./ml.)	Nitrogen (%)	Hexoses by orcinol method: glucose-mannose standard (%)	Hexose / Nitrogen
Dialysis of phenol solution: fraction soluble in water (B)	0·82	8·5	25·0	3
Precipitation of phenol solution by 1 vol. ethanol, ppt. dialyzed: fraction soluble in water (A I)	0·72	7·2	28·5	4

the protein with which it remains in combination in this fraction of the seromucoid.

Purification of the water-soluble fraction after dialysis of the ethanol-phenol precipitate (A I). Two methods were employed, (1) repeated precipitation by ethanol, and (2) agitation with chloroform.

(1) *Ethanol precipitation.* Since a trial fractionation by addition of 1, 2 or 3 vol. of ethanol did not lead to a fraction with appreciably augmented H/N ratio, the material was precipitated twice by 5 vol. of ethanol, the final precipitate being completely soluble in water. This fraction A II is characterized by an increase of the H/N ratio to 5.

(2) *Agitation with chloroform.* A solution (approx. 0·5 % (w/v)) of the above material A II was shaken repeatedly with chloroform and a few drops of amyl alcohol as described by Sevag (1934). In the first series of experiments the reaction was neutral. When a gel of protein was no longer formed, the material was precipitated by ethanol and redissolved in water yielding fraction A III in which the H/N ratio had been raised to 10. The chloroform treatment was continued with fraction A III at an acid reaction (pH 4–5, by addition of HCl) until a thin layer of protein no longer formed at the interface. Fraction A IV was thus obtained which, although containing hexose, contained so little nitrogen that, with quantities available, determinations fell within the range of error of the micro-Kjeldahl method. The ratio H/N was subsequently determined upon larger quantities of material (fraction C I, see below), and found to be 17. The above results are collected in Table 5.

Table 5. *Further fractionation of the carbohydrate-rich fraction of seromucoid. Hexoses determined by orcinol method*

Manipulations	Fraction	$\frac{\text{Hexose}}{\text{Nitrogen}}$
	Seromucoid	1·04
Solution in phenol, precipitation by 1 vol. ethanol, dialysis	A I	4
Two successive reprecipitations of A I by 5 vol. ethanol	A II	5
Repeated shaking of A II with chloroform and amyl alcohol in neutral solution	A III	10
Repeated shaking of A III with chloroform and amyl alcohol in solution at pH 5	A IV	17

Preparation and analysis of two fractions resulting from seromucoid by phenol cleavage

The application of the results of the small scale experiments to a larger quantity of seromucoid, yielded two fractions C I and C II as follows (see scheme, Fig. 3):

Seromucoid (1 g.) was ground in a mortar with 10 ml. of 92 % (w/v) phenol and the mixture shaken and left for some hours at room temperature, when all dissolved. An equal volume of absolute ethanol was then added and the insoluble fraction separated, suspended in water and dialyzed until free from phenol. The precipitate was collected on the centrifuge, washed with water, dissolved in the minimal quantity of 0·05 N-NaOH, then neutralized and again dialyzed, affording fraction C II.

The aqueous mother liquor, to which were added the water washings from the preparation of C II, was treated with 5 vol. of absolute ethanol, the precipitation repeated, the material dissolved in 30 ml. of water, and shaken repeatedly with 6 ml. portions of chloroform until a protein gel no longer formed. The collected chloroform phases were washed with water to remove any non-coagulated protein, and these washings themselves treated with fresh chloroform as above until gel no longer formed, and the aqueous residue united with the main fraction. To the aqueous solution was then added 5 vol. of ethanol, the resulting precipitate was

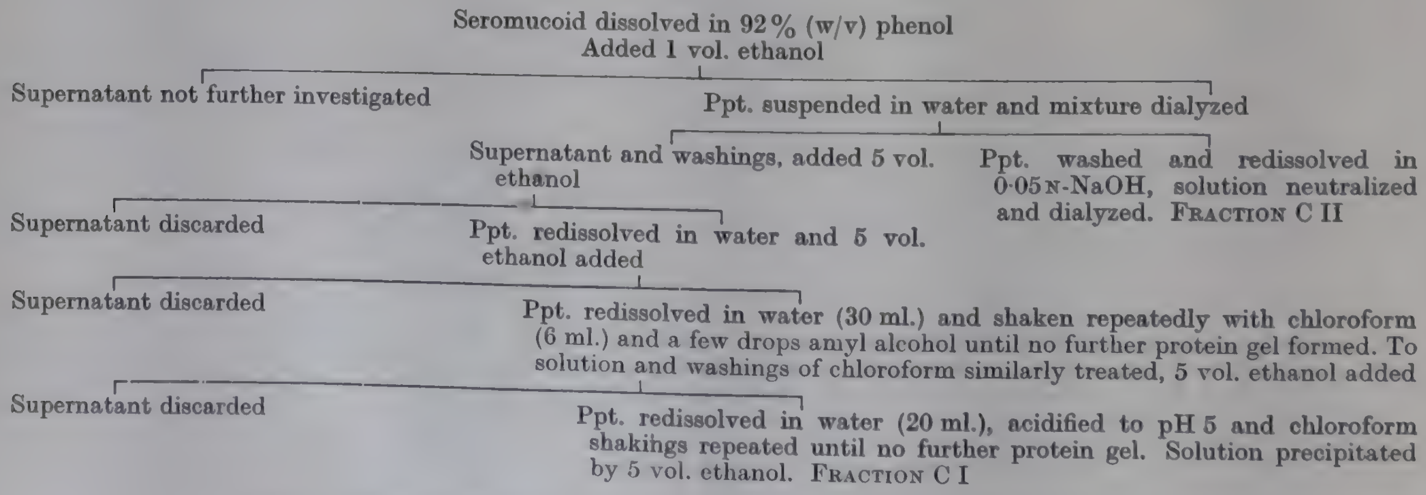

Seromucoid dissolved in 92 % (w/v) phenol
Added 1 vol. ethanol
Supernatant not further investigated
Ppt. suspended in water and mixture dialyzed
Supernatant and washings, added 5 vol. ethanol
Ppt. washed and redissolved in 0·05 N-NaOH, solution neutralized and dialyzed. FRACTION C II
Supernatant discarded
Ppt. redissolved in water and 5 vol. ethanol added
Supernatant discarded
Ppt. redissolved in water (30 ml.) and shaken repeatedly with chloroform (6 ml.) and a few drops amyl alcohol until no further protein gel formed. To solution and washings of chloroform similarly treated, 5 vol. ethanol added
Supernatant discarded
Ppt. redissolved in water (20 ml.), acidified to pH 5 and chloroform shakihgs repeated until no further protein gel. Solution precipitated by 5 vol. ethanol. FRACTION C I

Fig. 3. Scheme of fractionation of seromucoid by phenol and chloroform.

redissolved in 20 ml. of water, acidified to c. pH 5 and again treated with chloroform as already described. A final precipitation with ethanol, resolution and dialysis afforded fraction C I.

Table 3 records the analytical findings upon fractions C I and C II with, for comparison, those derived from the fractions obtained by Na_2SO_4 precipitation.

DISCUSSION

A study of the curves A (nitrogen : concentration of Na_2SO_4), B (hexoses : concentration of Na_2SO_4) and C (nitrogen : hexoses) of Fig. 1 shows that the greater part of the seromucoid is precipitated between the limits of 225 and 425 mg./ml. of Na_2SO_4, the latter figure being close to the figure for full saturation at 37° (440 mg./ml.). It also shows that although the seromucoid studied was not a pure substance (the relationship in curve C would in that case have been represented by a straight line) the amount of impurity was relatively small (compare KN with the total curve, Fig. 1 A). To facilitate discussion, we will consider consecutively the four portions of the curves, corresponding to Na_2SO_4 concentrations of 200–275, 275–350 and 350–550 mg./ml., viz. QN, NK and KP, and finally the fraction of the glycoprotein not precipitated by Na_2SO_4.

QN. Our results in this region are indefinite owing to the very fine nature of the precipitates which form in small quantity and are difficult to retain by the G 3 filters employed. On curve C the portion NQ may be co-linear with the mean experimental values grouped about N, but curves A and B, on the other hand, might suggest that the protein precipitating in this region differs from that precipitating subsequently. Nevertheless the differences between the determinations are often only slightly greater than the limit of experimental error of the methods, and it is not possible to form an unequivocal conclusion concerning this small portion of the seromucoid studied.

NK. All three curves show that over this region it is possible to speak of a characteristic glycoprotein constituting the main fraction of the seromucoid (containing 70% of the nitrogen and 40% of the hexose of the whole material) and having a ratio H/N of 0·72 (orcinol method) or 1·00 (carbazole method) calculated from curve C.

Electrophoretic analysis at pH 8 of this fraction afforded some confirmation of its homogeneity (Fig. 4), but one must not overlook the fact that the electrophoresis was carried out at one pH only, and furthermore that it is possible by this method to overlook impurities which comprise less than 10% of the main material.

KP. The striking difference in the character of the curves A and B over this region and the much steeper slope of C clearly indicate the presence of a glycoprotein richer in carbohydrate than that previously precipitating and having a ratio H/N of 1·0 or 1·4 according to whether orcinol or carbazole is used for determination.

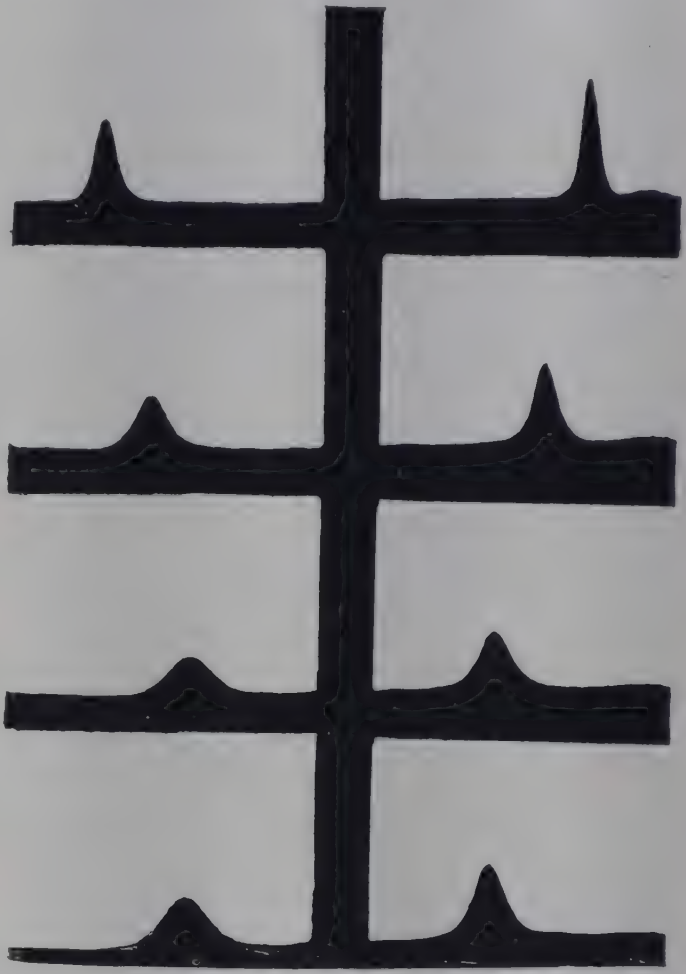


Fig. 4. Electrophoretic pattern of the main fraction obtained from seromucoid by salting out with sodium sulphate. Phosphate buffer, pH 8·0, ionic strength 0·2.

Residual fraction. A fraction representing approx. one-third of the total hexoses and a lesser fraction (approx. one-twelfth) of the total nitrogen remains unprecipitated by Na_2SO_4, the ratio H/N of this residual material being greater than 3.

An analytical study of fraction Na I (precipitation up to N) and Na III (precipitation from M to full saturation) agreed with expectations based upon deductions from the three curves (see Table 3). Unfortunately a laboratory mishap deprived us of the supposedly pure fraction Na II (precipitation between N and M) and we were unable in this instance to confirm by analysis the ratio $H/N = 1{\cdot}0$ deduced from the straight line MN. The value found for Na I (0·94) does not differ greatly from this figure, but we are unable to say whether the slight deviation is due to experimental error or to the presence here of a glycoprotein different from Na II and slightly poorer than it is in hexoses. On the other hand the difference between the value of 1·0 for Na II and 1·15 found for Na III is much beyond the possible limits of experimental error and agrees well with the values deducible from curve C.

It is possible indeed to calculate a theoretical value for the ratio H/N for fraction Na III knowing the values of H/N for the portions MK (1·00) and KP (1·4) and the relative proportions of these two materials in the fraction Na III, viz. 2·5/2 deduced from inspection of curve C.

One finds

$$H/N \times 4{\cdot}5 = 2{\cdot}5 \times 1{\cdot}0 + 2 \times 1{\cdot}4, \text{ i.e. } H/N = 1{\cdot}17.$$

The actual value of the ratio H/N, found by experiment was 1·15, an excellent agreement.

In addition to the above results, the study of Table 3 allows interesting conclusions to be drawn concerning the ratio of glucosamine to hexoses in the different fractions. One finds, indeed, an enrichment in glucosamine in Na I and Na III relative to the original seromucoid (0·57 and 0·58 respectively as against 0·45). Moreover these values are the same within the limits of experimental error as that afforded by the fraction C II obtained by phenol cleavage. This enrichment in glucosamine necessitates an impoverishment in the residual fraction not precipitated by Na_2SO_4, if one supposes, as seems reasonable, that fraction Na II would possess the same ratio as Na I and Na III (both of which contain more or less Na II). It would have been interesting to know whether the glucosamine : hexose ratio of the residual fraction, which unfortunately was not kept, was the same as that (0·15) of fraction C I obtained during fractionation with phenol.

It would appear, then, that the seromucoid which we studied contained different proteins united to two different polyosides, one of which was easily detachable by phenol (and perhaps by Na_2SO_4) and poor in glucosamine, whilst the other was more firmly bound to protein and relatively rich in glucosamine. Substantiation is thus afforded of the results obtained by Penet (1940) and Dumazert *et al.* (1943, 1944) and mentioned in the introduction to this paper. It seems indeed possible either that the presence of glucosamine in the easily hydrolyzable polyoside studied by them was not revealed on account of the very small quantities with which they worked, the glycoproteins being examined in the whole plasma, or that the small quantity was destroyed during hydrolysis.

Our determinations of hexose made with carbazole afforded in the case of fraction C I the red colour characteristic of glucose (which finding led us to use a reference standard of glucose while investigating this fraction), whilst all other fractions, viz. Na II, Na III, C II and seromucoid itself invariably gave the brown colour observable when the carbazole method is applied to galactose or to mixtures of glucose and mannose. The few μg. of tryptophan which would be present during these determinations made on glycoproteins could not be responsible for the difference in tint observed. This also accords with the results of Penet (1940) and Dumazert *et al.* (1943, 1944) who found that those carbohydrates which could be liberated by a mild hydrolysis were composed of glucose only.

A chromatographic study of hydrolysates of the two fractions C I and C II would doubtless permit an elucidation of the nature of the hexoses (Partridge, 1946) they contain; we were not able to do more than carry out orientating experiments in this direction owing to the departure of one of us having interrupted the work before definite conclusions could be reached.

CONCLUSION

We have separated from a seromucoid, previously prepared by one of us, a glycoprotein the solubility curve of which entitles it to be regarded as a pure substance, and also a fraction rich in carbohydrate but with a nitrogen content too high to allow it to be classed as a polyoside. This material resembles more closely the group of polyosido-polypeptides familiar to immunologists.

The carbohydrate moieties of these two fractions are distinctly different both as regards their glucosamine contents and the nature of their hexoses. In connexion with the latter one may recall the 'sucre virtuel' of Lépine (1921) which however has never up to the present time been isolated in the form of a polysaccharide from a glycoprotein. Penet (1940) stresses that it is not possible to affirm 'that it involves a linkage to protein'.

SUMMARY

1. Further investigation has been made of a seromucoid fraction previously prepared from ox blood.

2. Salting out studies with sodium sulphate at 37° showed that a fraction could be separated representing about 70 % of the nitrogen and about 40 % of the total bound hexose in the starting material, and satisfying the criterion of constant solubility and composition (hexose/nitrogen) demanded by a pure substance. The homogeneity of this fraction was supported by electrophoresis.

3. Cleavage of a fraction, rich in carbohydrate and very poor in nitrogen, from the seromucoid could be accomplished by fractionation with phenol-ethanol and repeated shaking with chloroform and amyl alcohol at suitable reactions. The residual material was correspondingly poorer in carbohydrate than the parent seromucoid.

4. The ratio of glucosamine to hexoses in these two fractions has been compared with that in the materials obtained by sodium sulphate fractionation. The particularly low ratio of glucosamine to

hexoses in the carbohydrate-rich fraction is emphasized.

5. The possibility is considered that the treatment of our seromucoid with sodium sulphate at 37° also results in the detachment of a carbohydrate-rich fraction.

6. The bearing of these preliminary findings upon current views concerning the nature of the serum glycoproteins is briefly discussed.

We wish to thank Dr R. A. Kekwick of the Lister Institute of Preventive Medicine for kindly performing the electrophoretic examination. One of us (A.M.S.) was the holder of a Fellowship from the French Government and Pasteur Institute during the course of this work.

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The Reduction of Methaemoglobin in Red Blood Cells and Studies on the Cause of Idiopathic Methaemoglobinaemia

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It is well established that methaemoglobin produced in the blood of normal animals by the injection of drugs disappears quite rapidly on withdrawal of the drug (Dennig, 1900; Stadie, 1921), and that its disappearance is due to reactions taking place in the red cell itself (Cox & Wendel, 1942). The last named authors showed that reduction of methaemoglobin taking place *in vivo* continued at the same speed in the shed blood, and did not depend on reducing substances in the plasma. Drug methaemoglobinaemias in man appear to be closely similar to those in animals, the foreign pigment usually disappearing within 24 hr. after the last dose of the drug.

In idiopathic methaemoglobinaemia, a rare disease which may be familial, it has been found that there is little or no tendency for the methaemoglobin to disappear from the shed blood (Lian, Frumusan & Sassier, 1939; Barcroft, Gibson, Harrison & McMurray, 1945). This observation has recently been confirmed by Sievers & Ryon (1946) who showed that the stability of the methaemoglobin probably does not depend on an abnormality of the plasma, since plasma from their patient did not affect the rate of reduction of methaemoglobin in normal human erythrocytes, nor did normal plasma alter the behaviour of methaemoglobin in erythrocytes from the case.

The work described here has been carried out on blood from six persons belonging to two families in which idiopathic methaemoglobinaemia occurred. It has been possible to obtain evidence of a specific deficiency in the enzyme system of erythrocytes from the cases, and also to draw conclusions concerning the reactions involved in the reduction of methaemoglobin in the blood of normal persons. Apart from their application to the study of idiopathic methaemoglobinaemia, the results are of interest since they throw light on the mechanism by which the haemoglobin of normal persons is maintained in a functional state. The mechanism of regeneration of haemoglobin from methaemoglobin is of importance as it has been suggested that methaemoglobin is constantly being formed in the blood (Peters & Van Slyke, 1931), and Paul & Kemp (1944) have shown that methaemoglobin is present in small amounts in normal human blood.

METHODS

Materials. Hexosediphosphate was prepared by the method of Morgan & Robison (1930). For use, the neutral Ba salt was rubbed up in a small glass mortar with the theoretical amount of N-H_2SO_4, and neutralized with NaOH after removal of $BaSO_4$. Hexosemonophosphate (Robison ester) was isolated from a yeast fermentation mixture as described by DuBois & Potter (1943). Final purification was effected through the Pb salt as described by Robison (1922). As the salt is soluble, Ba was removed at pH 8 as the phosphate. The acid Ba salt of D-3-phosphoglyceric acid was prepared from hexosediphosphate as described by Neuberg & Vercellone (1935). The purity of the specimen of the crystalline salt was checked by the polarimetric method of Meyerhof & Schulz (1938). Na pyruvate was prepared from the freshly distilled acid by the method of Peters (1938) and recrystallized twice from aqueous acetone. Diphosphopyridine nucleotide (DPN) was prepared from brewer's yeast by the method of Green & Brosteaux (1936). Later the isolation of larger quantities from baker's yeast was undertaken, using the simplified method of Green & Williamson (1940). The DPN content of the preparations was determined manometrically by measuring the CO_2 evolution on reduction with hydrosulphite in bicarbonate solution (Warburg, Christian & Griese, 1935). Coenzyme factor was prepared from ox heart in the form of the Straub flavoprotein (Straub, 1939). Purification was carried to the stage of elution from alumina C_γ.

Analytical methods. Venous blood samples of 30–50 ml. were taken from the subjects 2 hr. after breakfast and defibrinated by stirring with glass wool. Before use, the cells were washed three times with 5 vol. 0·9% (w/v) NaCl containing 0·01 M-phosphate buffer pH 7·4, (phosphate saline). It is important that the cells be washed with saline shortly after the blood is drawn, otherwise rapid reduction of methaemoglobin (MHb) may be observed in the controls without substrate.

In the experiments with normal blood and in some of the experiments with blood from the cases of idiopathic methaemoglobinaemia, it was necessary to form MHb in the cells. This was effected by treating the red cells with 3 vol. 0·075% (w/v) amyl nitrite in phosphate saline. The mixture was allowed to stand for 10–15 min. at room temperature, and the cells freed from excess nitrite by repeated washing with phosphate saline. This treatment converts about half of the haemoglobin to MHb. It was noted that in the absence of phosphate the formation of MHb by amyl nitrite takes place much less rapidly. The total pigment in the cell suspensions was usually about 14 g./100 ml. All results, however, have been corrected to refer to a suspension containing 15·6 g./100 ml. to facilitate comparison of the figures in the tables.

The reduction of MHb in the red cells was followed by measuring the uptake of CO consequent on the reduction of MHb to haemoglobin. Samples of 2 ml. of the red cell suspension were measured into the main chamber of Barcroft-Dixon flasks of about 40 ml. capacity. 0·2 ml. 2N-KOH and a roll of filter paper were placed in the inset and substrate added, after equilibration in the water bath, from Keilin tubes. The flasks were filled with 20% (v/v) CO in N_2 by repeated evacuation, the CO being prepared from formic and sulphuric acids. The results in all cases were corrected for the condensation of CO with the KOH in the insets to form formate (Warburg & Christian, 1931). The temperature in all experiments was 37°.

Haemolysates were prepared by washing red cells with 0·9% (w/v) NaCl, and cooling the concentrated corpuscular cream to $-2°$. Two vol. of ice-cold water were then added, and the mixture allowed to stand for 10 min. A volume of 0·5M-phosphate buffer pH 7·4 equal to that of the blood corpuscles was added and the cloudy liquid centrifuged at high speed for 15 min. The clear supernatant fluid was sucked off and used in the manometers. Pyruvic acid was determined on trichloroacetic acid filtrates of blood by the method of Long (1942). Phosphoglyceric acid was determined by the method of Rapoport (1936). When required, MHb was determined by the method of Evelyn & Malloy (1938), and total pigments by the method of Clegg & King (1942), standardized according to Gibson & Harrison (1945). Where necessary, the quantities of reagents taken for these colorimetric estimations were slightly altered to suit the Hilger Spekker absorptiometer.

RESULTS

Glucose and lactate as substrates in the reduction of methaemoglobin in erythrocytes

The reduction of methaemoglobin (MHb) produced by the action of nitrite on red cells from normal persons is slow when they are suspended in saline. The reduction is greatly accelerated by the addition of glucose (Table 1, cols. A, B). This confirms previous observations of Warburg, Kubowitz & Christian (1930), using rabbit erythrocytes. The effect of lactate is closely similar (Table 1, cols. A, C). Glucose and lactate together do not produce additive effects, which suggests that some stage in the overall reaction is common to both substrates. In the case of lactate it is found that the net molecular ratio *pyruvate formed/MHb reduced* is approximately 0·5 (Table 1, cols. G–E/C–A), in agreement with the equation

$$\text{lactate} + 2\text{MHb} = \text{pyruvate} + 2\text{Hb}. \qquad (1)$$

With glucose as substrate, the position is less clear, since there are several possible pathways for the reaction. The main problem is whether glucose acts before splitting, during glycolysis, into 3-carbon compounds, or after the splitting has taken place. In the first case the reactions would be of the type described by Dickens (1938), involving phosphorylation followed by step-wise oxidation of the molecule through phosphogluconic acid to phosphoketohexonate and decarboxylation to phosphopentose (Fig. 8, steps 1 and 2). The enzymes catalyzing these reactions have been shown to occur in horse blood and require coenzyme II. The other possibility is that reduction of MHb takes place after splitting of the glucose molecule to two triosephosphate molecules. The reactions possible would then be

$$\text{triosephosphate} + 2\text{MHb} = \text{phosphoglycerate} + 2\text{Hb}, \qquad (2)$$

Table 1. *The reduction of methaemoglobin in nitrite-treated erythrocytes from normal persons and from cases of idiopathic methaemoglobinaemia*

(Each manometer contained 2·0 ml. cell suspension, total pigment 15·6 g./100 ml. Cells suspended in 0·9% (w/v) NaCl made 0·01 M with phosphate buffer pH 7·4. Substrates added from Keilin tubes at the end of the equilibration period. Reaction period 4 hr. at 37°, gas phase 20% (v/v) CO in N_2. KOH papers in insets. Final concentrations, glucose 0·01 M, lactate 0·02 M.)

	MHb reduced (µmol.)			Pyruvate (µmol.)				Ratio Net pyruvate / Net MHb	
Normal bloods	Cells A	Cells + glucose B	Cells + lactate C	Cells initial D	Cells final E	Cells + glucose F	Cells + lactate G	Cells + glucose H	Cells + lactate I
1	1·0	3·9	3·9	0·5	3·6	2·4	5·1	0·49	0·52
2	0·9	3·0	3·5	0·3	2·1	1·8	3·3	0·50	0·46
3	0·8	3·6	4·1	0·3	3·5	2·0	5·5	0·47	0·49
4	1·0	3·2	—	0·4	2·9	2·0	—	0·50	—
5	1·2	2·7	3·0	0·5	2·4	1·8	3·4	0·48	0·55
6	2·0	6·0	6·6	0·5	4·4	3·8	6·8	0·53	0·52
Cases									
1	0·7	1·6	0·8	0·2	3·3	1·0	3·5	—	—
2	0·5	0·8	0·7	1·0	4·0	0·9	4·0	—	—
3	0·7	0·8	0·7	0·4	2·8	0·4	2·9	—	—
4	0·4	0·6	0·7	0·2	3·2	0·5	3·4	—	—
5	1·0	1·7	1·7	—	—	—	—	—	—
6	1·2	1·8	1·7	—	—	—	—	—	—

or glycolysis to lactate may be completed and reaction (1) follow. These reactions require coenzyme I. It is unfortunately impossible to test these reactions directly by trying out the intermediates postulated since the envelope of the erythrocyte is impermeable to the phosphorylated compounds, and the reduction of MHb effectively ceases on haemolysis.

Some indirect evidence is however available that reactions (1) and (2) are principally concerned.

(*a*) In glycolysis the oxidation of triosephosphate is coupled with the reduction of pyruvate so that the latter part of the scheme may be written

$$\begin{tikzcd}
\text{triosephosphate} \arrow[r, "\text{oxidation}"] & \text{phosphoglycerate} \arrow[d] \\
\text{lactate} & \text{pyruvate} \arrow[l, "\text{reduction}"']
\end{tikzcd}$$

Owing to the coupling of triosephosphate and pyruvate in the oxido-reduction, there can be no increase in the sum of the phosphoglycerate and pyruvate unless either triosephosphate or lactate is oxidized by some reaction outside the scheme. If the reduction of MHb takes place at the expense of either triosephosphate or lactate, then an increase in phosphoglycerate or pyruvate will occur. The figures in Table 1, col. H, i.e. cols. (F–D)/B, show that an amount of pyruvate equivalent to the amount of MHb reduced is formed in normal erythrocytes during the reduction of MHb by glucose. Net values, however, cannot be obtained by comparison with controls without substrate, because red cells contain a large amount of phosphoglycerate (Jost, 1927), which breaks down in the absence of glucose to give pyruvate (Table 1, cols. D, E). The cells from the cases of methaemoglobinaemia may be used as controls, since in them little reduction of MHb is taking place, and no accumulation of pyruvate occurs in the presence of glucose, while in the absence of glucose, pyruvate is formed in an amount similar to that found in normal cells (Table 1, cols. D, F, and D, E).

(*b*) In the presence of fluoride, the breakdown of phosphoglycerate is inhibited (Lohmann & Meyerhof, 1934), so that reduction of MHb by means of reaction (2) would be accompanied by the formation of an equivalent amount of phosphoglycerate. It has been found that reduction of MHb in the presence of glucose is not inhibited by 0·01 M-NaF, and that in its presence phosphoglycerate accumulates to a significant extent only when MHb is being reduced (Table 2). In the estimation of phosphoglycerate by the method of Rapoport (1936) it was found that with blood there was a tendency to give a greyish brown colour as compared with the blue of the standard, probably due to charring of organic material during heating with H_2SO_4 (Meyerhof & Schulz, 1938). For this reason, and also because of the large number of subtractions needed to obtain net values for comparison with the amounts of MHb reduced, close stoichiometric equivalence was not obtained, but the figures show that both MHb and glucose are required for substantial formation of phosphoglycerate.

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Table 2. *The formation of phosphoglycerate in normal erythrocytes during the reduction of methaemoglobin in the presence of* 0·01 M-NaF

(Conditions as in Table 1, except that each manometer contained, in addition, 0·01 M-NaF. Incubation for 3 hr., except for the manometer containing methylene blue, which was incubated for 1 hr. only.)

	Phosphoglycerate (µmol.)		
	Washed cells	Nitrite-treated cells	MHb reduced (µmol.)
Initial	2·2	2·2	—
Final	2·8	3·2	2·5
Final + glucose	3·4	5·9	4·7
Final + glucose + 5×10^{-5} M-methylene blue	—	6·1	12·0

(*c*) Monoiodoacetate is known to inhibit the triosephosphoric enzyme. If MHb reduction depends on the oxidation of triosephosphate, it would be expected that iodoacetate would have an inhibitory effect, and, as shown in Table 3, it has been found that small concentrations of iodoacetate produce a 90% inhibition of MHb reduction. This effect is not due to a non-specific inhibitory action of iodoacetate, since reduction of MHb by lactate is unaffected.

Table 3. *The effect of iodoacetate on the reduction of methaemoglobin in normal erythrocytes in the presence of glucose and lactate*

(Conditions as for Table 1.)

	MHb reduced (µmol.)			
	Gross		Net	
	Control	Iodoacetate 0·002 M	Control	Iodoacetate 0·002 M
Cells	0·8	0·8	—	—
Cells + glucose	3·5	1·0	2·7	0·2
Cells + lactate	4·0	4·0	3·2	3·2

Reduction of methaemoglobin by glucose and lactate in cells from cases of idiopathic methaemoglobinaemia

It has been found (Table 1, cols. A, B, C; Fig. 1) that there is relatively little tendency for MHb to disappear from the red cells of cases of idiopathic methaemoglobinaemia either in the presence or absence of glucose or lactate, and that there is correspondingly little formation of pyruvate during the experimental period (Table 1, cols. D, F and E, G). As the work on normal cells which has just been described had suggested that MHb reduction under these conditions depends chiefly on the triosephosphoric and lactic enzymes, the cells from the

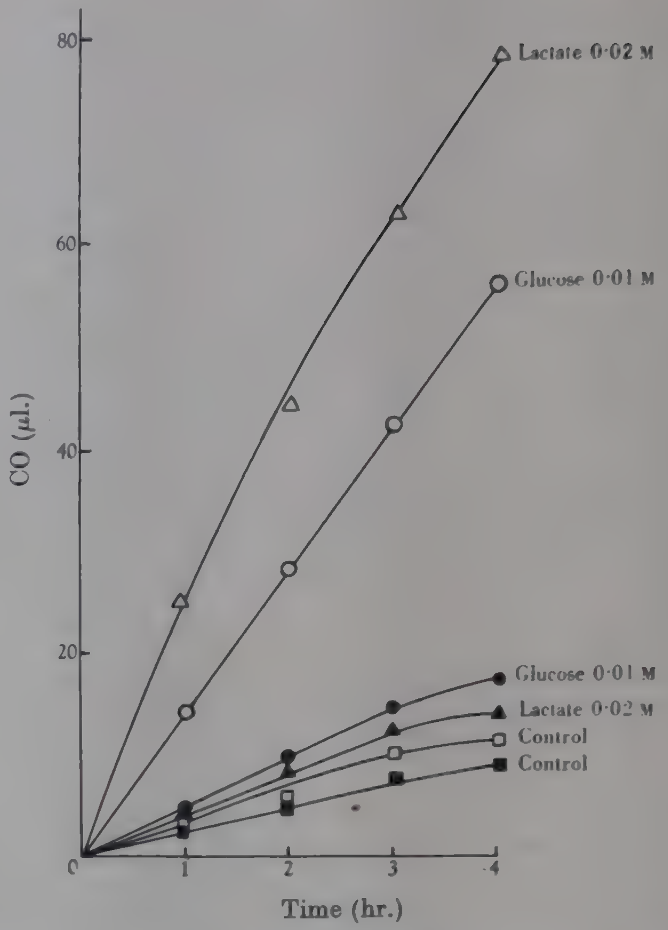

Fig. 1. Reduction of MHb by erythrocytes from a normal person and from a case of idiopathic methaemoglobinaemia. The ordinate gives the amount of MHb reduced, expressed in µl. CO absorbed by 2·0 ml. of a cell suspension containing 15·6 g./100 ml. total pigments, incubated at 37°. The clear symbols refer to the normal, the shaded symbols to the case. The conditions are the same as in Table 1.

Table 4. *The disappearance of pyruvate from normal erythrocytes and from erythrocytes from cases of idiopathic methaemoglobinaemia*

(Values given are µmol. pyruvate present/vessel. Each contained 2·0 ml. cell suspension in 0·9% (w/v) NaCl containing 0·01 M-phosphate buffer, pH 7·4. Total pigment 15·6 g./100 ml., 0·1 ml. 0·5 M-NaF, 0·2 ml. 0·029 M-Na pyruvate, and 0·2 ml. 0·1 M-glucose, or 0·2 ml. 0·9% (w/v) NaCl. Incubation at 37° in air for 1 hr.)

	Pyruvate (µmol.)		
Normals	Initial	Final	Final + glucose
1	6·8	6·4	2·4
2	6·8	6·3	2·0
3	6·8	6·4	3·0
4	6·8	6·5	2·0
5	6·8	5·8	3·0
Cases			
1	6·8	6·3	2·0
2	6·8	6·4	2·8
4	6·8	6·2	2·6

cases were investigated for a possible deficiency of these enzymes. To test this point, the disappearance of pyruvate in the presence of glucose and NaF was measured in normal and pathological cells. This reaction (Dische, 1937) involves both the triosephosphoric and lactic enzymes and diphosphopyridine nucleotide (DPN). The results given in Table 4 show that there is no significant difference, in this respect, between the normal and pathological cells. The cells from the cases are therefore not deficient in these enzymes and DPN (Fig. 8 A, 1–4).

The effect of methylene blue on the reduction of methaemoglobin by glucose and lactate

The finding of active triosephosphoric and lactic enzymes in the cells from cases of idiopathic methaemoglobinaemia, suggested that the failure of these cells to reduce MHb might be due to a deficiency of some oxidation-reduction carrier intervening between the pyridine nucleotides and MHb. In view of the effect of methylene blue in accelerating the reduction of MHb *in vivo* (Cox & Wendel, 1942), its action *in vitro* was examined. It was found, using normal cells (Table 5), that small amounts of the dye accelerated the reduction of MHb by glucose more than ten times. The rate of reduction by lactate was approximately doubled. Dewan & Green (1938) have shown that the reduction of methylene blue by reduced DPN is catalyzed by 'coenzyme factor', while Corran, Green & Straub (1939) have shown that 'coenzyme factor' is probably identical with the flavoprotein isolated by Straub (1939) from heart muscle. In the light of these findings it is probable that methylene blue acts as a carrier between this or a similar flavoprotein and MHb.

Table 5. *Reduction of methaemoglobin in the presence of* 5×10^{-5} M-*methylene blue by cells from cases of idiopathic methaemoglobinaemia and by cells from normal subjects*

(The figures show the amount of MHb reduced/hour. In comparing with Table 1, it should be noted that the figures there are for a period of 4 hr. The conditions are as for Table 1, except that each manometer contained 5×10^{-5} M-methylene blue in addition.)

	MHb reduced (μmol./hr.)		
Normals	Cells	Cells + 0·01 M-glucose	Cells + 0·02 M-lactate
1	0·8	8·2	3·0
2	0·9	13·0	—
3	1·1	16·2	3·9
4	1·3	—	4·5
5	1·8	9·6	6·1
Cases			
1	0·7	12·7	1·0
2	0·4	11·8	0·6
3	0·3	10·5	0·5
4	0·4	12·2	0·8
5	0·8	15·7	1·0
6	0·5	19·2	0·8

The results with the pathological cells are in striking contrast to the results with normal cells (Table 5). In the abnormal cells, the rate of reduction in the presence of methylene blue and glucose is accelerated in a manner similar to that found in normal cells. With lactate, however (Table 5; Fig. 2), the rate of MHb reduction is scarcely changed on the addition of methylene blue. This finding is notably different from that in normal blood, where the rate of reduction with lactate is substantially accelerated.

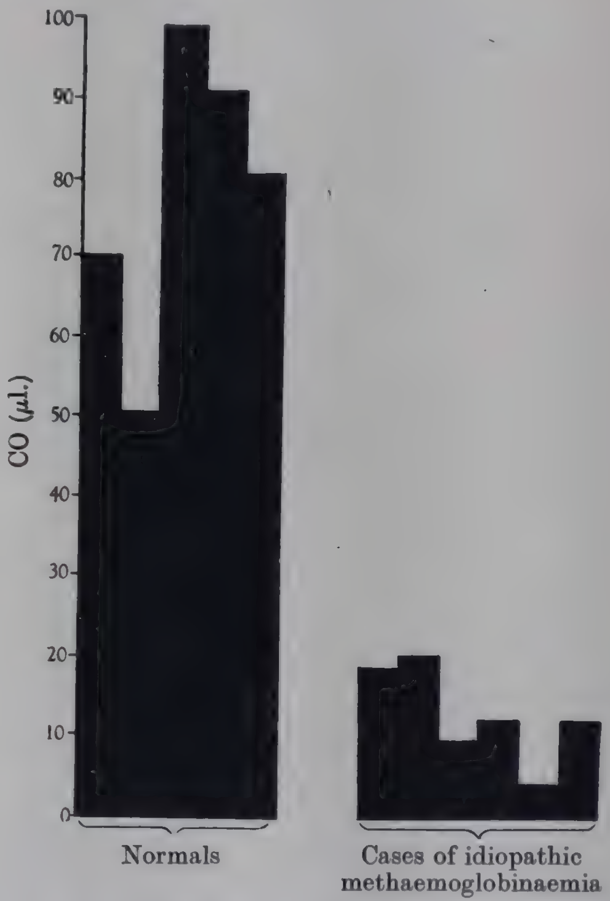

Fig. 2. MHb reduction by erythrocytes from normal persons and from cases of idiopathic methaemoglobinaemia in the presence of lactate and methylene blue. Each manometer contained 2·0 ml. nitrite-treated cell suspension (total pigment 15·6 g./100 ml.), 0·02 M-Na lactate and 5×10^{-5} M-methylene blue. The results are corrected for reduction of MHb taking place in controls without substrate. Temp. 37°, gas phase 20% (v/v) CO in N_2, reaction period 1 hr.

The pathway of the reaction in the presence of glucose and methylene blue is uncertain. The following evidence is available.

(*a*) The reaction does not appear to proceed via the triosephosphoric or lactic enzymes, since the amount of pyruvate accumulating is far below that which would be expected if lactate were the ultimate reductant of MHb (Table 6), while the amount of phosphoglycerate accumulating in the presence of fluoride is similarly too small to account for the methaemoglobin reduced (Table 2).

Table 6. *The relation between methaemoglobin reduced and pyruvate formed in the presence of glucose and methylene blue*

(Conditions as in Table 1, except that each manometer contained 5×10^{-5} M-methylene blue. The figures for MHb are gross quantities reduced, the figures for expected pyruvate are calculated on the assumption that 1 mol. pyruvate corresponds to 2 mol. MHb. Incubation 4 hr.)

Normals	MHb reduced (μmol.)	Pyruvate formed (*A*) (μmol.)	Pyruvate expected (*B*) (μmol.)	100 *A*/*B*
1	9·4	1·09	4·7	23
2	7·5	0·80	3·8	21
3	10·6	1·28	5·3	24
4	6·4	1·05	3·2	32
5	12·6	1·46	5·3	23
Cases				
1	7·6	0·60	3·8	16
2	17·0	1·72	8·5	20
3	4·8	0·44	2·4	18
4	5·5	0·87	2·7	32

(*b*) Phosphorylation of glucose probably precedes the reduction of MHb, since arsenate, which interferes with phosphate transfer in the oxidation of triosephosphate (Needham & Pillai, 1937), produces a marked inhibition of MHb reduction. Oxalate, which is believed to inhibit the transfer of phosphate from phosphopyruvate (Lohmann & Meyerhof, 1934), produces less inhibition than arsenate, but the effect of the two together is more than additive. An experiment of this type is summarized in Fig. 3.

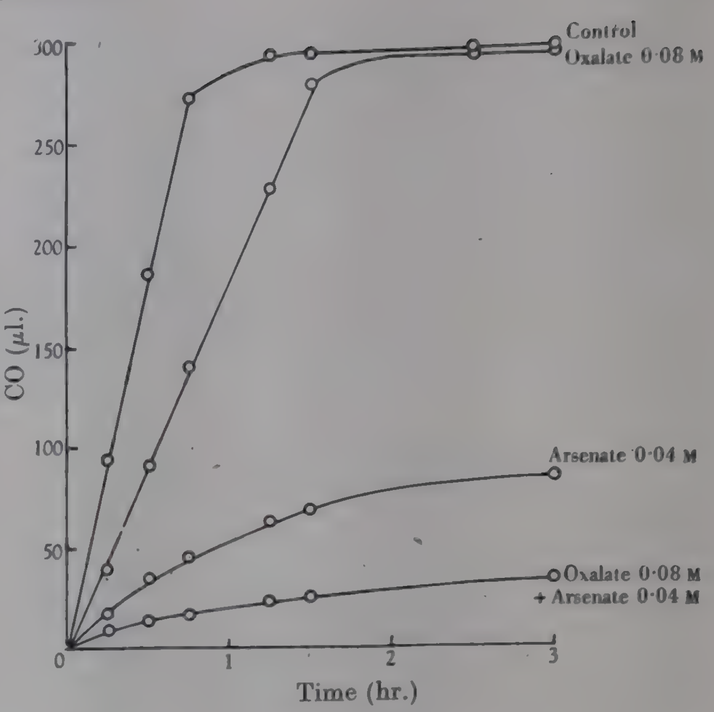

Fig. 3. The effect of arsenate and oxalate on the reduction of MHb in nitrite-treated erythrocytes in the presence of glucose and methylene blue. Each manometer contained 2·0 ml. cell suspension (total pigments 15·6 g./100 ml.), 0·01 M-glucose and 5×10^{-5} M-methylene blue. The glucose was added after equilibration at 37° from Keilin tubes. Gas phase 20% (v/v) CO in N_2.

(*c*) When minimal quantities of glucose are present, the amount of MHb reduced/mol. glucose added exceeds the theoretical maximum possible, if oxidation of glucose were taking place after splitting of the sugar molecule to two 3-carbon compounds. If triosephosphate or lactate were the substrates concerned, the maximum possible ratio would be 4 mol. MHb/mol. glucose. In the presence of fluoride or oxalate, it has been found that this ratio may be substantially exceeded. An experiment of this kind is shown in Fig. 4. It should be noted that ratios of this kind represent minimum values, since any loss of glucose by side reactions such as glycolysis would tend to depress them.

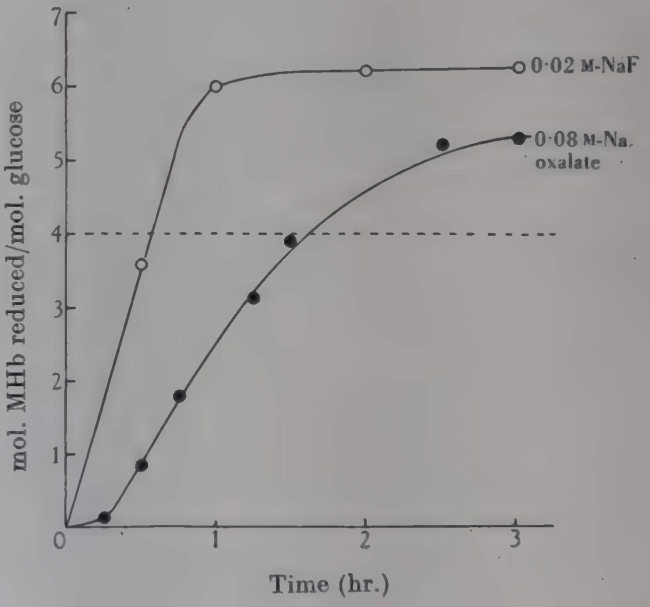

Fig. 4. The figure shows the amount of MHb reduced/mol. glucose added, after various periods of time. Each manometer contained 2·0 ml. nitrite-treated cell suspension in phosphate saline, 5×10^{-5} M-methylene blue, and 1 μmol. glucose which was added from a Keilin tube. The results are corrected for the reduction of MHb which took place in controls without added glucose. The theoretical maximum amount of MHb which could be reduced after splitting to two three-carbon compounds is indicated by the dotted line.

The marked difference between normal and pathological bloods in the presence of glucose only, and the similarity of their behaviour in the presence of glucose and methylene blue, suggests that the addition of methylene blue virtually opens up a new reaction pathway, rather than that it acts by accelerating a reaction which is already proceeding. These results may be accounted for by supposing that in the presence of methylene blue, stepwise oxidation of sugar occurs according to the scheme of Dickens (1938), who has shown that the necessary enzymes are present in the blood of the horse as well as in yeast (Fig. 8, col. B). In this step-wise breakdown of carbohydrate it would be possible for at least 8 mol. MHb to be reduced/mol. glucose used, and phosphorylation would precede the utilization of the sugar. Further, the enzymes in Dickens's (1938) scheme require triphosphopyridine nucleotide, as opposed to the enzymes taking part in the reduction of MHb in normal cells in the absence of methylene blue, which require DPN.

Coenzyme factor activity in normal red cells and in cells from cases of idiopathic methaemoglobinaemia

The finding that the cells from the cases of methaemoglobinaemia were unable to reduce MHb appreciably faster in the presence of lactate + methylene blue than in the presence of methylene blue alone (Table 5, Fig. 2) together with evidence that there are adequate amounts of the triosephosphoric and lactic enzymes in these cells (Table 4) suggested that the cells might be deficient in coenzyme factor activity. Measurements were therefore made of the rate of oxidation of triosephosphate in haemolysates prepared from normal blood and from the blood of the cases. It was found that under the conditions used (0·1 M-phosphate buffer, pH 7·4, 7×10^{-3} M-Na hexosediphosphate, 5×10^{-4} M-DPN, 2×10^{-4} M-methylene blue), the limiting factor governing the rate of oxidation of triosephosphate was the coenzyme factor when this was added in the form of a solution containing the Straub flavoprotein (Straub, 1939).

The results of assays of 'coenzyme factor' activity in twelve consecutive normal bloods and in the blood of the cases are given in Fig. 5, where the blackened portions correspond to the oxygen uptakes by the system specified. The total heights of the columns (shaded + unshaded) represent the uptakes in one-third of the time by the system + Straub flavoprotein where this uptake was determined.

The first three cases of methaemoglobinaemia examined gave results which are markedly lower than those obtained with normal blood. In the last two cases, however, the difference is much less striking. The assays of the first three cases were interpolated among assays of normal bloods, while the last two were carried out some time afterwards. The assays of the pathological bloods were in all cases repeated upon two separate blood samples taken at different times, with results which did not differ by more than 10%. After the assays of the last two cases had given higher results than for the other abnormal bloods, one of the earlier patients was recalled and the assay repeated. The result of this experiment confirmed the original low figure for this patient, so there does not appear to be any doubt either of the low figures for the first three cases examined, or of the higher figures for the last two. It should be noted that the last two cases were much less severely affected than the others, having initial blood MHb levels of less than 2 g./100 ml. while the first three had initial MHb levels ranging from 5·1 to 7·8 g./100 ml. The last two cases, nos. 5 and 6 in Table 1, also showed a higher enzyme activity in the

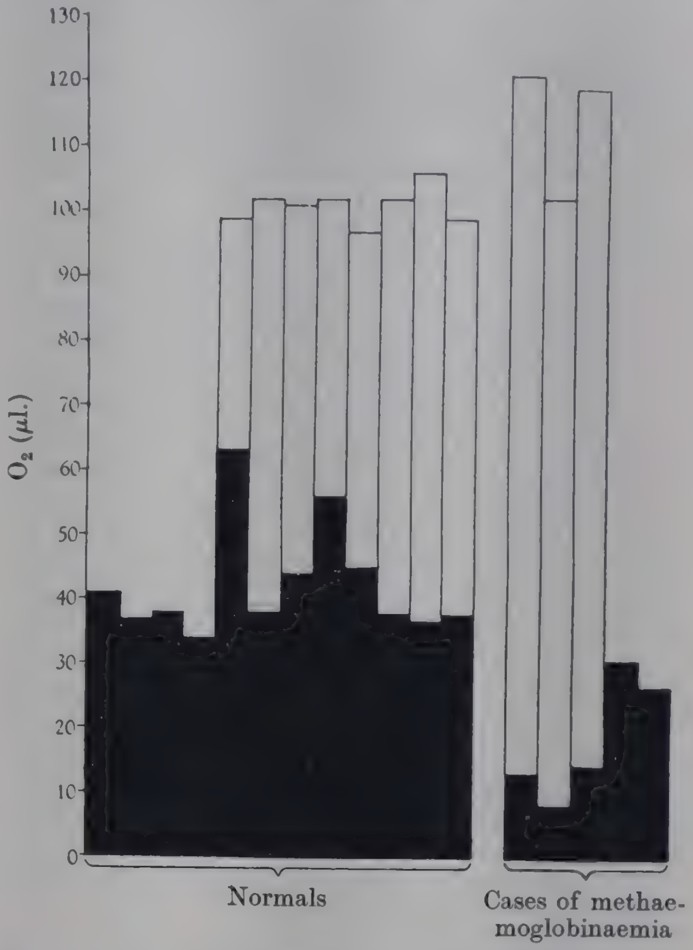

Fig. 5. The solid rectangles represent the amount of O_2 absorbed by 2·0 ml. of a haemolysate containing 5·6 g./100 ml. total pigment, in the presence of 0·1 M-phosphate buffer pH 7·4, 0·007 M-Na hexosediphosphate, 5×10^{-4} M-diphosphopyridine nucleotide, and 2×10^{-4} M-methylene blue. The heights of the whole columns (shaded + unshaded) represent the amount of O_2 absorbed by the same system in the presence of 0·4 ml. of a preparation of the Straub flavoprotein. The methylene blue was added from Keilin tubes. The figures for the shaded portions give the uptakes from 15 min. after the addition of the dye until 60 min. afterwards. The whole columns represent the uptakes in the first 15 min. after the addition of methylene blue. Incubation at 37° in air. In the last two cases examined the preparation of Straub flavoprotein had become relatively inactive. It produced a large increase in both cases, but the final figures were not considered reliable and so have been omitted.

intact cells. Taking the results as a whole, it would seem justifiable to conclude that a definite deficiency in the coenzyme factor activity of the blood of persons suffering from idiopathic methaemoglobinaemia has been demonstrated. The coenzyme factor which was tested in these experiments is coenzyme factor I reacting with DPN. The deficiency of this factor in the cells from the cases provides a satisfactory explanation of the failure of methylene blue to accelerate lactate oxidation in the intact pathological cells.

Correction required in coenzyme factor assays. In carrying out these experiments it was assumed that the rate of reaction between methylene blue and reduced DPN is negligible in the absence of flavoprotein, but later work showed that this is by no means true, and that the figures obtained required correction for the oxygen transferred by direct reaction between reduced DPN and methylene blue. As the size of this correction had an important bearing on the comparisons made between normal and abnormal bloods it was determined with some care. Reduced DPN was prepared by the method of Ohlmeyer (1938) and allowed to react with methylene blue in phosphate buffer in air at 37°. The reaction was followed manometrically. The results, plotting the logarithm of reduced DPN concentration against time for two different methylene blue concentrations, show that the reaction is apparently bimolecular (Fig. 6). This allows of the calculation of the correction for any required amounts of DPN and methylene blue.

(*a*) In the lactic enzyme system prepared from acetone powder of heart muscle extract, varying amounts of DPN were added in the presence of 0·065 M-HCN, 0·08 M-phosphate buffer pH 7·4, and 0·016 M-Na lactate. The results of several experiments gave a mean value for the correction of 28 μl./hr.

(*b*) Experiments in which varying amounts of DPN were added to blood haemolysates showed that the addition of the amount required to give a final concentration of 6×10^{-5} M approximately doubled the oxygen uptake, while further additions up to a concentration of 10^{-3} M gave uptakes which increased slowly and gradually beyond this point. These results suggested that the enzyme system was almost effectively saturated at the lower concentration and that the slow increase on further addition of DPN was due to the direct reaction between reduced DPN and methylene blue. The correction derived from these experiments was 26 μl./hr. These independent methods of determining the correction lead to practically the same result, and the average value has been used in correcting the coenzyme factor assays.

It will be noted that in Fig. 5 the oxygen uptakes have been recorded over a period of 45 min. which began 15 min. after the addition of methylene blue from a Keilin tube. The readings in the initial period were disregarded. This was done for two reasons, namely that in the controls without substrate, a small amount of MHb is formed by oxidation by the methylene blue, and that when the blood from the cases was used, which already contained a large amount of MHb, rapid reduction occurred as soon as the methylene blue was added and continued until an equilibrium amount of MHb was present. As the transfer of 1 electron will lead to the uptake of 1 mol. O_2 when applied to the reduction of MHb, whereas 4 electrons must be transferred when H_2O is the end product, it is desirable to postpone the initial reading until reasonable time has been allowed for equilibrium with regard to MHb, and the reaction has settled down to a steady rate.

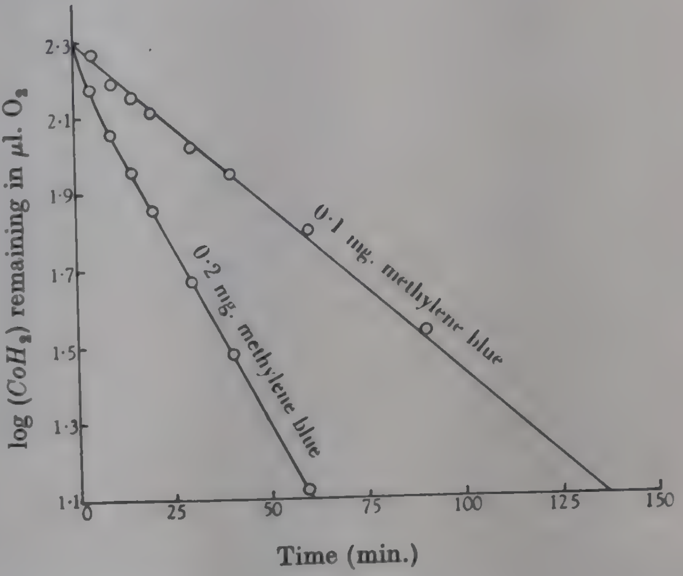

Fig. 6. Oxidation of reduced diphosphopyridine nucleotide by molecular O_2 in the presence of methylene blue. Each manometer contained 5·9 mg. reduced diphosphopyridine nucleotide (CoH_2), in 0·1 M-phosphate buffer, pH 7·4. The quantity of methylene blue shown on the graph was added from a Keilin tube after temperature equilibration. Temp. 37°, gas phase air. The ordinate shows the amount of reduced diphosphopyridine nucleotide present at any time expressed as the log. of the equivalent amount of O_2. As H_2O_2 is the end product under the conditions used, the figures are twice those which would be observed in the presence of catalase.

The oxidation of hexosemonophosphate in haemolysates

In view of the suggestion that the reduction of MHb in the presence of glucose + methylene blue may proceed by means of coenzyme II dehydrogenases, it was of interest to examine the oxidation of hexosemonophosphate in the blood of normal persons and in the blood of the cases. Owing to the difficulty of preparing coenzyme II, fully detailed studies were not carried out, but it was found, as would be expected if this suggestion is correct, that the rate of oxidation of hexosemonophosphate was substantially similar in both normal and abnormal bloods (Fig. 7).

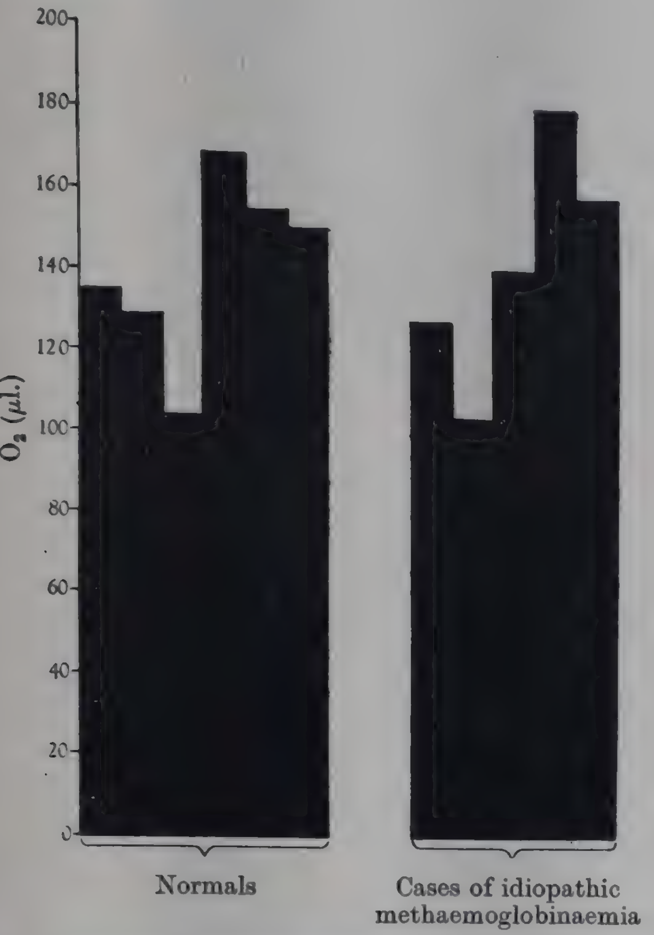

Fig. 7. Oxidation of hexosemonophosphate in haemolysates of normal blood and of blood from cases of idiopathic methaemoglobinaemia. Each manometer contained 2·0 ml. haemolysate (total pigment 5·6 g./100 ml.), 2×10^{-4} M-methylene blue, 5×10^{-4} M-diphosphopyridine nucleotide and 0·07 M-Na hexosemonophosphate. The methylene blue was added from Keilin tubes after temperature equilibration. The height of the columns represents the amount of O_2 absorbed in 1 hr. at 37° after the addition of the methylene blue. Gas phase air.

DISCUSSION

On the basis of the work which has been described, the following tentative explanation is offered for the occurrence of idiopathic methaemoglobinaemia, and of the normal mechanism for the reduction of MHb in the blood.

(*a*) MHb reduction requires coenzyme I dehydrogenases, particularly the lactic and triosephosphoric dehydrogenases. Associated with these is coenzyme factor I. It is assumed that coenzyme II dehydrogenases do not play any very significant part in the reduction of MHb under normal circumstances, probably because coenzyme factor II does not react with MHb.

(*b*) On the addition of methylene blue, which is capable of reacting rapidly with both coenzyme factors I and II and MHb, the reduction of MHb is greatly speeded up both by an acceleration of the existing pathway via coenzyme I dehydrogenases and the opening up of new pathways via coenzyme II dehydrogenases in Dickens's scheme. These reactions are presented schematically in Fig. 8.

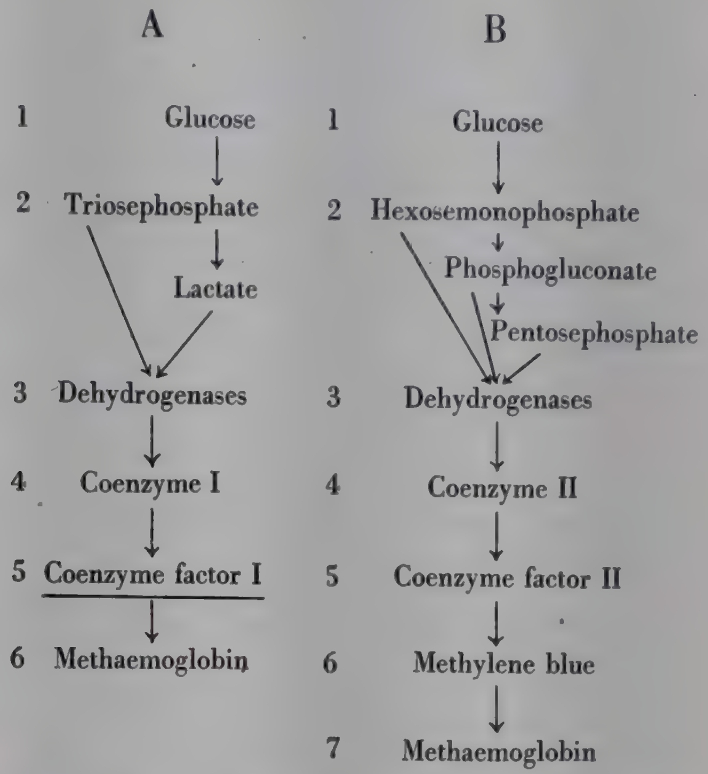

Fig. 8. Diagrammatic scheme of the reactions taking place in the reduction of MHb in erythrocytes. Column A reactions occurring in the absence of methylene blue; B in the presence of methylene blue. In cases of idiopathic methaemoglobinaemia where there is a deficiency of coenzyme factor I, the reactions in column A do not occur, and MHb reduction is very slow.

(*c*) MHb reduction fails to occur in the cases of idiopathic methaemoglobinaemia because of the deficiency of coenzyme factor I. The addition of methylene blue speeds up MHb reduction as with normal blood because of the presence of coenzyme II dehydrogenases and coenzyme factor II, together with glucose. In the presence of lactate, there is no marked effect on adding methylene blue because the lactic enzyme requires coenzyme I and coenzyme factor I which is present only in small amounts.

(*d*) MHb does not continue to increase in amount in the blood of persons affected with idiopathic methaemoglobinaemia to an unlimited extent, because, as the concentration of MHb rises, its rate of reaction with non-specific reducing agents in the blood will also rise and an equilibrium will be established. In this respect it is probable that ascorbic acid and glutathione are the most important reducing agents in the blood. The results of Gibson (1943) suggest that ascorbic acid reacts directly with MHb and the dehydroascorbic acid formed is again reduced by glutathione. The action of large amounts of ascorbic acid in bringing about a new equilibrium with much smaller amounts of MHb in the blood of these cases (Lian *et al.* 1939; Deeny, Murdock & Rogan, 1943; Barcroft *et al.* 1945; Sievers & Ryon, 1946) is probably due to a mass action effect of this kind.

It should be pointed out that direct evidence is not available for all the assumptions and suggestions made in this scheme. It has, however, the merit of accounting for all the reactions observed in the abnormal cells by means of a single deficiency, which has been demonstrated with reasonable assurance, namely the deficiency of coenzyme factor I. The difficulties met with in the work have been of two kinds: first, it has not been possible to obtain blood samples from the patients as often and at the times which would have been desirable, because none of the patients lived in Belfast, but had to travel for considerable distances to the laboratory; and secondly, it has been impossible to put the theories about the intermediates involved in the various reactions to a direct experimental test, since the red cells are impermeable to most of the substrates involved, and laking the cells stops the reduction of MHb.

There seems to be no doubt, however, that idiopathic methaemoglobinaemia belongs to the group of diseases due to an inborn error of metabolism and should be classified along with alcaptonuria and cystinuria rather than under enterogenous cyanosis. Of the alternative explanations possible for the accumulation of MHb in the blood of these cases, impermeability of the erythrocytes to glucose may be dismissed, since glucose is rapidly oxidized by the cells in the presence of methylene blue, while a rapid rate of formation of MHb also seems to be improbable, otherwise reducing systems would lead to the disappearance of the pigment from the shed blood as occurs in drug methaemoglobinaemias where the cells are normal. In this connexion it should be noted that Sievers & Ryon (1946) examined the blood of a case with great care for MHb-forming agents without finding any evidence of their presence.

The results bearing on the mechanism of MHb reduction in normal red cells are of considerable interest, since it is probable that MHb is constantly being formed in the blood of normal persons (Peters & Van Slyke, 1931). The low level which is present in normal blood, about 1 % of total pigment (Gibson & Harrison, 1945), would presumably represent the amount required to give equilibrium between the rates of formation and removal of the pigment. If there is such an equilibrium between formation and removal, the enzymatic mechanism described would account for the removal of MHb and the maintenance of the haemoglobin in a functional state in normal persons. Failure of any part of this enzyme system to function would lead to methaemoglobinaemia of the type exemplified by the patients described in this paper.

SUMMARY

1. Methaemoglobin reduction in normal erythrocytes takes place through the oxidation of triosephosphate and lactate.

2. The system requires coenzyme factor I in addition to the enzymes and coenzyme I.

3. Coenzyme II dehydrogenases do not play a part in the normal mechanism of methaemoglobin reduction, but on the addition of methylene blue, they take the principal part in bringing about the great acceleration of methaemoglobin reduction which is observed.

4. Methaemoglobin present in the blood of persons suffering from idiopathic methaemoglobinaemia is not reduced with appreciable speed in the presence of glucose or lactate.

5. Blood from cases of idiopathic methaemoglobinaemia contains less coenzyme factor I activity than does normal blood.

6. It is suggested that, owing to the deficiency of coenzyme factor I in the blood of patients suffering from idiopathic methaemoglobinaemia, the enzymatic reducing activity is lower than in normal cells, leading to a displacement of the equilibrium Hb $\rightleftharpoons$MHb to the right and the consequent accumulation of abnormal amounts of MHb in the blood.

The author wishes to thank Prof. D. C. Harrison for his interest in the work.

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Studies on Glyoxalase

1. A NEW FACTOR

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The experiments to be described took origin from a personal observation by one of us (E.J.M.). He found that the enzyme glyoxalase was well adsorbed on calcium phosphate gel at pH 5–6, the filtrate showing no activity at all in the presence of methylglyoxal and glutathione. When, however, a proportionate amount of the filtrate was added to the eluate from the calcium phosphate the activity of this was largely increased.

EXPERIMENTAL

General methods

The method was that used in our previous paper (Hopkins & Morgan, 1945), namely, the determination of the rate of CO_2 evolution from $NaHCO_3$ solution, due to lactic acid production from methylglyoxal. We employed Barcroft differential manometers provided with standardized flasks. Each flask contained a measured quantity of the enzyme solution or other preparations, 0·6 ml. of 0·2M-$NaHCO_3$, with 2 mg. of glutathione (GSH) in 0·3 ml. water in a Keilin cup. To one flask 2·1 mg. of methylglyoxal were added, and the total contents of each flask made up to 3 ml. with distilled water (any variation of these quantities is given in the text). The apparatus was filled with a gas mixture of 95 vol. N_2 + 5 vol. CO_2, the GSH tipped in and the manometer shaken in a bath at 25°. The stopcocks were left open for 4 min. and then closed.

The majority of our experiments were carried out with ox heart, as this was the most convenient tissue to obtain fresh in sufficient quantities. Acetone preparations of the hearts were prepared. In making these, owing to the scarcity of acetone, smaller quantities were used than usual. The hearts freed from fat were minced, and extracted at 0° with three times their weight of acetone for 30 min., filtered, and extracted a second time for 15 min., using half the previous volume of acetone. The extracted heart was filtered off and dried in large vacuum desiccators. It was then ground to a fine powder in a mill. This acetone heart powder if kept dry keeps its activity for several months.

Separation of the new factor from the enzyme

The following separation was repeated several times; the results are shown in Table 1.

1st stage. 80 g. of acetone heart powder were extracted by stirring with 400 ml. of distilled water for 1 hr. and then centrifuged. The supernatant, which was highly pigmented, measured 234 ml. Of this, 0·3 ml. (dry wt. 9·90 mg.) gave 303·2 μl. CO_2 in 20 min.

2nd stage. Fractional precipitation with ammonium sulphate. To 220 ml. of the supernatant, solid ammonium sulphate (68·64 g.) was slowly added to give 50 % saturation. After standing 2 hr. the precipitate was centrifuged off and discarded.

The filtered, clear supernatant measured 234 ml. The ammonium sulphate concentration was then raised to 70 % saturation by adding the calculated amount of the solid (31·6 g.). After standing overnight in the refrigerator, the precipitate, which contained both the factor and the enzyme, was filtered off on two ordinary funnels, and given one good wash by filling up the papers with 70 % saturated ammonium sulphate. The precipitate was scraped off the paper, dissolved in about 50 ml. of distilled water and dialyzed in cellophan tubing against distilled water, with frequent changes, for 18–24 hr. An inactive precipitate was centrifuged off and the supernatant diluted to 110 ml. (i.e. half the volume originally taken). The pH of this solution was approximately 5·4 and needed no adjustment for the next stage. 0·3 ml. (dry wt. 5·25 mg.) gave 302·5 μl. CO_2 in 20 min.

3rd stage. Adsorption of the enzyme. 100 ml. of the above solution were used for this stage. The enzyme was completely adsorbed on calcium phosphate gel suspension (30 mg. dry wt./ml.) by adding four successive lots of 10 ml., allowing each to stand 15 min. with an occasional stir, and then each time centrifuging off the gel. The supernatant contained the factor (see Table 1). 0·42 ml. (dry wt. 3·24 mg.), equivalent to 0·3 ml. of the 100 ml. taken for stage 3, gave 21·0 μl. CO_2 in 20 min.

Elution of the enzyme. The four lots of calcium phosphate gel containing the enzyme were mixed, and washed well by stirring with 200 ml. of distilled water to which was added 10 ml. of Sørensen's phosphate buffer pH 5·4. This washing was repeated three times. The enzyme was then eluted from the calcium phosphate by two successive extractions with phosphate buffer pH 7·8 containing 10 % (w/v) ammonium sulphate, 50 and 20 ml. respectively being added. The two eluates were mixed, dialyzed for 24 hr., made up to 100 ml. and a very small inactive precipitate filtered off. Of the enzyme solution 0·3 ml. (dry wt. 1·5 mg.) gave 123·3 μl. CO_2 in 20 min.; 0·3 ml. enzyme + 0·42 ml. of factor gave 279·0 μl. CO_2 in 20 min. Other results are shown in Table 1. Preparations 1 and 2 were made from separate supplies of hearts.

It will be seen from the results (Table 1) that the presence of the factor has in each case more than doubled the velocity of action of the enzyme alone, the average increase being 141·7 %. In each case the enzyme and factor are equivalent to the same weight of heart powder.

Table 1. *Evidence for the new factor*

Preparation	Acid production from methylglyoxal (μl. CO_2 evolved from $NaHCO_3$ in 20 min.) By 0·3 ml. enzyme	By 0·42 ml. factor	By 0·3 ml. enzyme + 0·42 ml. factor
1	82·2	21·0	183·0
1	75·0	22·0	216·0
1	74·0	18·0	228·0
1	96·0	21·0	186·0
1	95·1	21·0	246·0
2	138·0	23·1	294·0
2	74·5	22·0	216·0
2	115·6	15·4	246·0
Mean	93·8	20·4*	226·8

* This small reading is not of enzymic origin, but due to some chemical action between GSH and methylglyoxal (Jowett & Quastel, 1933; Hopkins & Morgan, 1945).

Nature of the action of the factor

It is apparent from Fig. 1 that the effect of the factor is accelerative, maximal CO_2 liberation with enzyme + factor being attained in 20 min.; with enzyme alone in 140 min. The stopcocks were open in this experiment for 5 min., therefore the μl. of CO_2 evolved during this time, which we may take from the curves to be 60·0 and 25·27 respectively, must be added to the amount evolved (Fig. 1) affording totals for the enzyme + factor of 282·7 and for the enzyme alone of 282·0 μl.

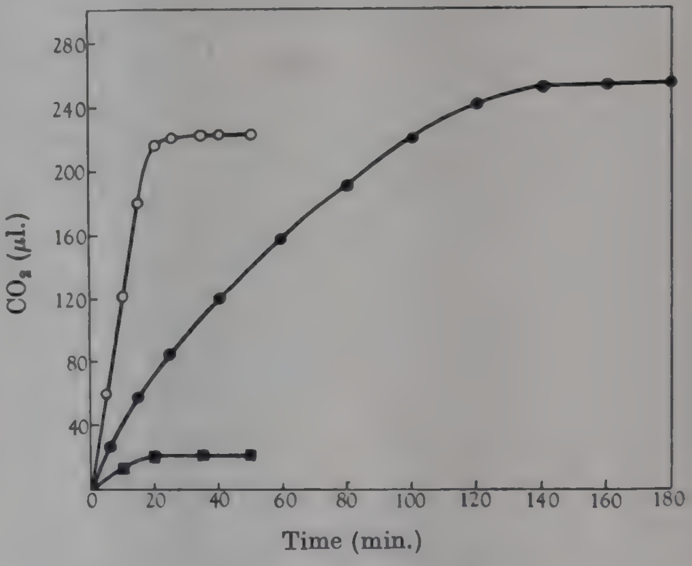

Fig. 1. Effect of factor on the action of glyoxalase. Curve ●—●, 0·3 ml. enzyme; ■—■, 0·42 ml. factor; ○—○, 0·3 ml. enzyme + 0·42 ml. factor. 1·0 mg. methylglyoxal and 2·0 mg. GSH in each. The dry weight of 0·3 ml. enzyme was 1·65 mg. and of 0·42 ml. factor 0·82 mg.

Further purification and properties of the factor

The factor was further purified by the following three additional stages.

4th stage. To 112 ml. of the supernatant (stage 3) 45·9 g. of ammonium sulphate were slowly added giving a final concentration of 63 % saturation. After standing 2 hr. the precipitate was filtered off and washed with 63 % saturated ammonium sulphate. The precipitate was scraped off the filter paper, dissolved in distilled water, dialyzed 18 hr. and made up to 80 ml. (this is the same dilution as the finish of stage 2). Of this solution 0·3 ml. (dry wt. 0·82 mg.) + 0·3 ml. enzyme gave 267·0 μl. CO_2 in 20 min.

5th stage. The solution was adjusted to pH 7·3 with sodium bicarbonate and heated in a water bath to 55° for 1 min. The solution was quickly cooled and the coagulum centrifuged off. Of the supernatant 0·3 ml. (dry wt. 0·38 mg.) + 0·3 ml. enzyme gave 237·0 μl. CO_2 in 20 min. It will be seen that there was some loss in activity of this particular preparation by heating. In all subsequent preparations there was no loss by heating to 55°.

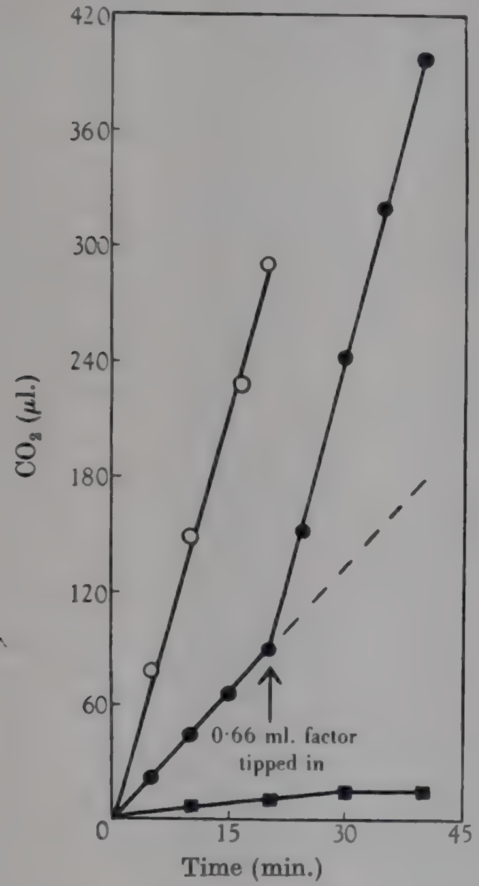

Fig. 2. Promptness of action of the factor. Curve ○—○, 0·3 ml. enzyme + 0·66 ml. factor; ●—●, 0·3 ml. enzyme, with 0·66 ml. factor tipped in at arrow; ■—■, 0·66 ml. factor, 2·0 mg. GSH and 2·1 mg. methylglyoxal in each. The dry weight of 0·3 ml. enzyme was 1·0 mg., and of 0·66 ml. factor 0·308 mg.

6th stage. 50 ml. were adjusted to pH 5·4 with dilute acetic acid and 5 ml. of calcium phosphate gel added (in later preparations 20 ml. were used). After standing 15 min. the calcium phosphate was centrifuged off and discarded. 0·33 ml. (dry wt. 0·154 mg.) + 0·3 ml. enzyme gave 201·0 μl. CO_2 in 20 min. For the dry weight of the 5th and 6th stage, measured portions were dialyzed. At this stage the solution was crystal clear. The factor can be concentrated by precipitating with ammonium sulphate (final concentration 63 % saturated) taking the precipitate up in the minimal quantity of distilled water and dialyzing until free from ammonium sulphate. Working on a much larger scale the solutions were kept more concentrated after the 2nd stage. One large scale attempt to crystallize the factor from ammonium sulphate failed, the activity decreasing, probably due to the acidity of the ammonium sulphate. In the following experiments we used the purified factor.

Action of factor immediate. The factor prepared as above exerts its full effect immediately it is added to the system, i.e. without lapse of time for any interaction with the reactants; this is shown in Fig. 2.

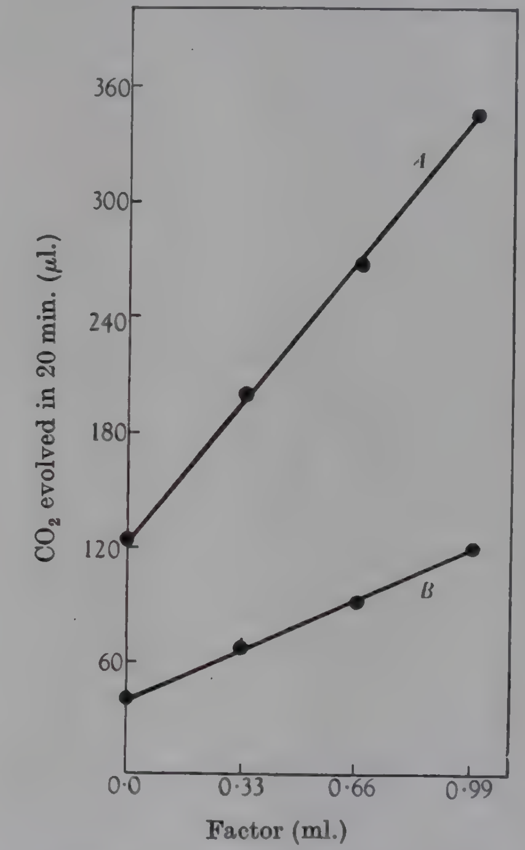

Fig. 3. Effects of varying quantities of the factor on glyoxalase action. 0·3 ml. of enzyme in each Barcroft flask, with varying amounts of factor. Dry weights the same as Fig. 2. Curve *A* with 2·0 mg. GSH, and curve *B* with 0·25 mg. GSH; 2·1 mg. methylglyoxal in each.

Variation of velocity of reaction with factor concentration. Fig. 3 shows the effect on the velocity of reaction of increasing the concentration of the factor with a constant volume of enzyme.

Dependence of action by the factor upon presence of GSH. Three Barcrofts were set up as follows: *A*, one flask with 0·3 ml. enzyme (1·0 mg.) + 0·6 ml. factor (0·2 mg.) + GSH but no methylglyoxal; the other flask with 2·4 ml. water + 0·6 ml. bicarbonate; *B*, with enzyme + factor + methylglyoxal but no GSH;

C, with enzyme + factor + methylglyoxal + GSH. There was no CO_2 evolution in *A* and *B*, whilst in *C*, 207·0 μl.CO_2 were evolved in 20 min.

Experiments with phenylglyoxal as the substrate

Using preparations of glyoxalase as ordinarily made, the velocity of the reaction was very much slower with phenylglyoxal than with methylglyoxal. Equimolar quantities of the substrates were used. The results are shown in Table 2. The enzyme preparations were made by grinding 10 g. of tissue with 30 ml. of water in a mortar for 30 min., using a little sand, and then centrifuging. The supernatant was dialyzed for 24 hr., made up to 50 ml. and filtered. 0·3 ml. of the preparations were used in the Barcroft.

Table 2. *Methyl and phenylglyoxals as substrates*

Tissue	Time (min.)	CO_2 evolved from $NaHCO_3$ (μl.) With 4·0 mg. phenylglyoxal	With 2·1 mg. methylglyoxal
Ox heart	20	77·1	135·0
Ox liver	10	90·0	287·8
Rat liver	10	72·0	225·0
Rat erythrocytes	10	108·0	237·0
Sheep liver	10	90·0	315·0
Pig liver	20	180·0	309·0
Acetone ox heart	20	99·0	300·0
Acetone yeast	20	138·0	223·6

Relation of the factor to phenylglyoxal. In Table 3 it will be seen that when the enzyme is separated from the factor, the former acts with about equal velocity on methyl- and phenylglyoxal. When, however, the factor is added to the enzyme, it greatly increases the velocity of reaction with methylglyoxal whereas in the case of phenylglyoxal it produces no effect.

It will be seen from the following table (Table 4) that phenylglyoxal combines with the factor. On adding phenyl and methylglyoxal in equimolar proportions to the enzyme + factor (Exp. G) there was

Table 3. *Differential effect of the factor on reaction with methylglyoxal*

Substrate	CO_2 (μl.) evolved in 20 min. from $NaHCO_3$, with	
	0·3 ml. enzyme	0·3 ml. enzyme + 0·42 ml. factor
(*a*) Methylglyoxal, 2·1 mg.	96·2	186·0
Phenylglyoxal, 4·0 mg.	102·0	96·0
	0·3 ml. enzyme (1·5 mg.)	0·3 ml. enzyme + 0·2 ml. factor (0·2 mg.)
(*b*) Methylglyoxal, 2·1 mg.	79·6	156·0
Phenylglyoxal, 2·0 mg.	74·2	66·0
	0·3 ml. enzyme (1·0 mg.)	0·3 ml. enzyme + 0·66 ml. factor (0·308 mg.)
(*c*) Methylglyoxal, 2·1 mg.	123·3	267·0
Phenylglyoxal, 4·0 mg.	120·0	118·2
	0·3 ml. enzyme (0·9 mg.)	0·3 ml. enzyme + 0·6 ml. factor (0·6 mg.)
(*d*)* Methylglyoxal, 2·1 mg.	54·0	163·6
Phenylglyoxal, 2·0 mg.	41·0	45·0

* 0·5 mg. GSH.

Table 4. *Combination of the factor with phenylglyoxal*

(The reactants were measured into the Barcroft flasks in the following order: water, bicarbonate, 0·3 ml. enzyme, 0·6 ml. factor, methylglyoxal, phenylglyoxal, and 0·5 mg. GSH tipped in. Dry weights in experiments A–I: 0·3 ml. enzyme, 0·99 mg.; 0·6 ml. factor, 0·6 mg. J–L, and M and N, contained different preparations of the enzyme and factor. The final concentrations of the substrates are given in brackets.)

Exp.	Reaction mixture	CO_2 evolved in 20 min. (μl.)
A	Enzyme + 2·1 mg. methylglyoxal (0·01 M)	41·1
B	+ factor + 2·1 mg. methylglyoxal (0·01 M)	105·0
C	+ 4·0 mg. phenylglyoxal (0·01 M)	41·1
D	+ factor + 4·0 mg. phenylglyoxal (0·01 M)	42·0
E	+ 2·0 mg. phenylglyoxal (0·005 M)	42·0
F	+ 1·0 mg. phenylglyoxal (0·0025 M)	36·0
G	+ 4·0 mg. phenylglyoxal (0·01 M) + 2·1 mg. methylglyoxal (0·01 M)	42·0
H	+ 2·0 mg. phenylglyoxal (0·005 M) + 2·1 mg. methylglyoxal (0·01 M)	66·0
I	+ 1·0 mg. phenylglyoxal (0·0025 M) + 2·1 mg. methylglyoxal (0·01 M)	74·5
J	+ 2·1 mg. methylglyoxal	123·0
K	+ 2·0 mg. phenylglyoxal	56·5
L	+ 2·0 mg. phenylglyoxal dialyzed 24 hr. + 2·1 mg. methylglyoxal	111·0
M	+ 2·1 mg. methylglyoxal	156·0
N	+ 2·1 mg. methylglyoxal + 1·125 mg. mandelic acid	154·0

no increase in velocity; lowering the amount of phenylglyoxal so that the methylglyoxal was in the proportion of 2 to 1 (Exp. H) gave an increase of 24 μl. CO_2 evolved, and a 4 to 1 ratio (Exp. I) gave 32·5 μl. This shows that phenylglyoxal has the greater affinity for the factor but the velocity of its enzymic decomposition is not increased as is the case with methylglyoxal. That the phenylglyoxal can be dialyzed away from the factor is shown by Exps. J, K and L. Mandelic acid does not inhibit in concentrations that would be formed during the experiments (Exps. M and N).

The factor is protein in nature, or closely associated with a protein. The solutions give good biuret and ninhydrin reactions, and positive colour tests for tyrosine, tryptophan, arginine, histidine and cystine.

The purest preparation of the factor we have so far prepared, when used in a quantity of 0·1 mg. with 1·0 mg. of enzyme, increased the velocity of reaction by 66·6 %. The activity was completely destroyed by heating at 70° for 3 min.

DISCUSSION

It will be seen from the results of the experiments described above that when methyl- and phenylglyoxal are added to a preparation of glyoxalase as ordinarily made from a tissue, the velocity of the reaction with the former is often more than three times that with the latter substrate (Table 2).

Lohmann (1932), in his experiments showing that glutathione was the coenzyme for glyoxalase, obtained this effect with either (he did not state which) rat or rabbit liver.

There seems to be no doubt that this increased velocity, when methylglyoxal is the substrate, is due to the new factor which we have separated and purified. We have shown that when the enzyme and factor are separated, the velocity of the reaction with the enzyme alone is the same for both substrates. But when the factor is added to the enzyme it is only the methylglyoxal which shows increased velocity, the phenylglyoxal giving the same evolution of CO_2 as for the enzyme alone (Table 3). This is shown to be due to the fact that, although phenylglyoxal combines with the factor, such combination does not increase the velocity of reaction (Table 4). We have emphasized earlier (Fig. 1) that the essential effect of the presence of the factor is an accelerative one.

In respect of the separation of the enzyme from the factor, it was successful enough in that solutions of the latter show no trace of enzyme activity. It is possible that the partially purified preparations of the enzyme as described, might leave it in association with small quantities of the factor, and that these are responsible for, and essential to, the action of the enzyme on methylglyoxal. It seems to us that strong evidence against this is yielded by the identical velocity of reaction displayed when methyl- and phenylglyoxal react with our preparations of the enzyme. Since the factor has no effect in the case of phenylglyoxal, it would if present as an impurity secure a greater velocity for the methylglyoxal. Furthermore, as the factor is completely inhibited by phenylglyoxal (Table 4), any traces left in the enzyme solution would also be inhibited, therefore it follows that if the enzyme needed the factor for its activity, phenylglyoxal would not react to give an evolution of CO_2.

We hope that further work, which is in progress, may elucidate the role of the new factor.

SUMMARY

1. A preliminary purification of glyoxalase is described.

2. A new factor, present in tissues, is shown to accelerate the conversion of methylglyoxal to lactic acid.

3. Phenylglyoxal inhibits the factor.

4. The factor has been separated and purified.

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Anti-histamine Effect of Sodium Salicylate and its Bearing upon the Skin-diffusing Activity of Hyaluronidase

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It was claimed by Guerra (1946 *a*, *b*) that the administration of sodium salicylate to rabbits in a dosage of 0·1 g./kg. body weight inhibited the skin-diffusing activity of hyaluronidase to a significant extent. Swyer (1948), on the other hand, failed to find any inhibitory effect of salicylates on the viscosity-reducing activity of hyaluronidase except when the concentration of the former reached such high levels that the accompanying reduction of pH itself caused inhibition of the enzyme action. In an attempt to account for this discrepancy between the *in vivo* and *in vitro* results, it was suggested that the hyaluronidase preparation used by Guerra might have contained histamine, or some substance with a similar action, which, by increasing the capillary permeability, would have enhanced the spreading effect of the hyaluronidase. The further suggestion was made that salicylate might have acted as a histamine antagonist, thereby reducing the spreading action of the hyaluronidase preparation. In order to investigate the above suggestion, a study, the results of which are reported in this paper, was made of the effect of salicylate on the skin-diffusing activity of hyaluronidase prepared from bull semen and of snake venom, together with its effect on the capillary-damaging action of these substances, and also of histamine alone and when mixed with hyaluronidase.

METHODS

Two experiments, similarly planned, were made: the first tested the spreading action and capillary-damaging effect of bull-testis hyaluronidase and snake venom before and after the administration of salicylate; the second tested the spreading effect of hyaluronidase and of hyaluronidase together with histamine, and the capillary-damaging action of histamine alone, before and after the administration of salicylate. In each experiment three albino rabbits weighing between 2 and 3·3 kg. were used. They were depilated several hours before the commencement of the experiment by means of a barium sulphide paste. Each rabbit received an intravenous injection of pontamine blue, 5% (w/v) in saline, 0·6 ml./kg. body weight, followed 15 min. later by the intradermal injection in 0·2 ml. quantities of the substances to be tested, the injections being confined to the left flank. Two injections of each substance were made in each rabbit, four substances being tested at a time, the arrangement of injections being completely randomized in eight approximately square areas marked out on the rabbits' flanks. Injections containing India ink in approximately 10% concentration were used to show up the spreading action, while injections devoid of India ink were used to demonstrate the capillary-damaging effect, manifested by the appearance of the blue dye. The average diameters of the areas of blue dye or India ink respectively were measured 30 min. after the intradermal injections. The rabbits were then given intravenous injections of sodium salicylate in 10% (w/v) solution, each receiving 0·1 g./kg. body weight. After 15 min., the intradermal injections were repeated on the right flank, the results being read after a further 30 min. as before.

In Exp. 1 the substances tested were bull-semen hyaluronidase, 10 units (Swyer & Emmens, 1947) in 200 ml. saline (0·1 ml. + 0·1 ml. saline or 0·1 ml. India ink 20% v/v in saline) and rattlesnake venom, 14 mg. in 40 ml. saline, this solution having the same viscosity-reducing activity as that of the bull hyaluronidase (0·1 ml. + 0·1 ml. saline or 0·1 ml. India ink). The arrangement of injections is illustrated in Table 1.

Table 1. *Illustration of arrangement of intradermal injections in Exp.* 1

(*a*) Mixtures

Substance	Symbol
Hyaluronidase + saline	1, 3
Hyaluronidase + India ink	2, 4
Snake venom + saline	5, 7
Snake venom + India ink	6, 8

(*b*) Positions

Head

Before salicylate		After salicylate	
7	6	8	1
3	2	7	3
5	8	5	6
1	4	2	4

In Exp. 2 the substances tested were bull-semen hyaluronidase as in Exp. 1 containing 20% (v/v) India ink (0·1 ml. + 0·1 ml. saline); histamine diphosphate 0·1 mg./ml. (0·1 ml. + 0·1 ml. saline); hyaluronidase + histamine (0·1 ml. of each); and saline (0·2 ml.).

Reading the results was complicated by the difficulty of separating, in some of the cases, the effect produced by the spread of India ink from that of staining with pontamine blue caused by increased capillary permeability. It was decided, therefore, to measure in each case the maximum blue area regardless of whether due to India ink or pontamine blue. That this was a satisfactory criterion of the effects under consideration is shown by the analysis of variance (Tables 3 and 7).

RESULTS

Experiment 1. *Bull-testis hyaluronidase and snake venom, before and after salicylate*

The mean diameters of stained areas produced by these substances, and read as described above, are shown in Table 2. The analysis of variance for this experiment is set out in Table 3, from which it may be concluded that (1) there are significant differences in the reactions of different rabbits; (2) the responses to hyaluronidase and to snake venom show highly significant differences, and (3) the overall effect of salicylate is highly significant. In addition, the differential response with India ink and with pontamine blue to hyaluronidase is significantly different from that to snake venom, as is the differential response before and after salicylate to the two substances.

In order further to analyze these differential responses, the results were split so as to furnish analyses of variance for hyaluronidase and for snake venom separately; these are set out in Tables 4 and 5 respectively. From Table 4 it can be seen that the difference in response of pontamine blue and India ink to hyaluronidase is highly significant; in other words, as appears from Table 2, the hyaluronidase preparation had no significant effect on the pontamine blue reaction. The difference in response to hyaluronidase before and after the administration of salicylate is seen not to be significant. In the case of the response to snake venom, Table 5 shows that there was again a significant difference of response of pontamine blue and of India ink, though reference to Table 2 shows that snake venom, in contrast to the hyaluronidase preparation, produced a marked pontamine blue reaction. There was, moreover, a highly significant reduction of response to snake venom after the administration of salicylate.

Table 2. *Mean diameters in millimetres of stained skin areas in Exp.* 1

	Hyaluronidase + saline		Hyaluronidase + India ink		Snake venom + saline		Snake venom + India ink	
Rabbit	Before salicylate	After salicylate	Before salicylate	After salicylate	Before salicylate	After salicylate	Before salicylate	After salicylate
1	0	5	12	7	24	20	20	17
	7	8	9	8	27	18	15	13
2	4	0	7	5	20	(14)	11	7
	0	0	9	5	22	12	10	9
3	0	0	10	6	24	22	15	10
	0	0	6	9	22	9	15	7
Totals	11	13	53	40	139	95	86	63

The value (14), missing from the data, was replaced by the method of least squares and one degree of freedom was dropped in the analysis of variance.

Table 3. *Analysis of variance for Exp.* 1

Source of variation	Degrees of freedom	Sum of squares	Mean square	*F*	*P*
Rabbits (A)	2	188·5417	94·2709	11·98	<0·001*
Hyaluronidase *v.* snake venom (B)	1	1474·0834	1474·0834	187·31	<0·001*
Pontamine blue *v.* India ink (C)	1	5·3334	5·3334	—	—
Before salicylate *v.* after salicylate (D)	1	126·7501	126·7501	16·11	<0·001*
		Interactions			
A/B	2	18·2916	9·1458	1·16	>0·05
A/C	2	3·7916	1·8958	—	—
A/D	2	6·1249	3·0625	—	—
B/C	1	494·0832	494·0832	62·78	<0·001*
B/D	1	65·3332	65·3332	8·30	>0·001,* <0·01
A/B/C	2	14·0414	7·0207	—	—
A/B/D	2	8·0418	4·0209	—	—
A/C/D	2	5·3751	2·6876	—	—
B/C/D	1	27·0002	27·0002	3·43	>0·05
A/B/C/D	2	9·1252	4·5626	—	—
Error	23	181·0000	7·8696	—	—
Total	46	2627·6667	—	—	—

* Highly significant.

1948

Table 4. *Analysis of variance for responses to hyaluronidase in Exp.* 1

Source of variation	Degrees of freedom	Sum of squares	Mean square	F	P
Rabbits (A)	2	54·25	27·125	5·76	>0·01, <0·05
Pontamine blue *v.* India ink (C)	1	198·375	198·375	42·13	<0·001*
Before salicylate *v.* after salicylate (D)	1	5·0416	5·0416	1·07	>0·05
		Interactions			
A/C	2	14·25	7·125	1·51	>0·05
A/D	2	7·5834	3·7917	—	—
C/D	1	9·3751	9·3751	1·99	>0·05
A/C/D	2	9·2499	4·6250	—	—
Error	12	56·5	4·7083	—	—
Total	23	354·625	—	—	—

* Highly significant.

Table 5. *Analysis of variance for responses to snake venom in Exp.* 1

Source of variation	Degrees of freedom	Sum of squares	Mean square	F	P
Rabbits (A)	2	152·5833	76·2917	6·74	>0·01, <0·05
Pontamine blue *v.* India ink (C)	1	301·0416	301·0416	26·60	<0·001*
Before salicylate *v.* after salicylate (D)	1	187·0416	187·0416	16·53	>0·001, <0·01*
		Interactions			
A/C	2	3·5834	1·7917	—	—
A/D	2	6·5834	3·2917	—	—
C/D	1	18·3751	18·3751	1·62	>0·05
A/C/D	2	5·2499	2·6250	—	—
Error	11	124·5000	11·3182	—	—
Total	22	798·9583	—	—	—

* Highly significant.

If one accepts the pontamine blue reaction as a reliable criterion of increased capillary permeability, the conclusion to be reached from the above results is (1) that a satisfactory comparison has been made of the action on capillaries and the skin-diffusing activity of hyaluronidase prepared from bull semen and of snake venom; (2) that salicylate had no effect on the skin-diffusing activity of hyaluronidase but did significantly reduce both the capillary-damaging and the skin-diffusing activities of snake venom.

Experiment 2. *Hyaluronidase alone and with histamine, before and after salicylate*

In view of the results obtained in Exp. 1, it was considered worth while to compare the spreading action of hyaluronidase with a hyaluronidase-histamine mixture, both before and after the administration of salicylate, since it seemed probable that part at least of the responses to snake venom could be attributable to the presence of histamine in the venom. By means of the technique previously described and used in Exp. 1, it was possible to test at one and the same time the effect of histamine, alone and in combination with hyaluronidase, on capillary permeability before and after the administration of salicylate.

The results of this experiment, given as mean diameters of stained areas, are shown in Table 6. The analysis of variance is set out in Table 7, from which it may be concluded (1) that differences between rabbits were significant, and (2) that the overall effects of hyaluronidase, histamine, and salicylate, were all highly significant. Furthermore, there was no significant interaction between histamine and hyaluronidase, which implies that their effects were strictly additive; the interaction between hyaluronidase and salicylate was significant but not great, while, in contrast, that between histamine and salicylate was highly significant. The somewhat artificial reduction in the sum of squares used as the error term by the inclusion of the six pairs of zeros contributed by saline alone accounts for the significance of the hyaluronidase and salicylate interaction. The other significant effects would, of course, remain significant with a much larger error term.

It is therefore to be concluded that the spreading action of hyaluronidase is increased in the predicted manner by the presence of histamine, and that, as in Exp. 1, salicylate scarcely influenced the spreading action of hyaluronidase, while, as can be seen by

Table 6. *Mean diameters in millimetres of stained skin areas in Exp.* 2

	Hyaluronidase + India ink		Hyaluronidase + histamine + India ink		Histamine + saline		Saline	
Rabbit	Before salicylate	After salicylate	Before salicylate	After salicylate	Before salicylate	After salicylate	Before salicylate	After salicylate
1	9	12	17	6	17	0	0	0
	7	9	30	11	19	0	0	0
2	9	9	25	12	19	0	0	0
	9	9	22	14	19	0	0	0
3	6	7	17	6	15	0	0	0
	8	10	20	7	14	0	0	0
Totals	48	56	131	56	103	0	0	0

Table 7. *Analysis of variance for Exp.* 2

Source of variation	Degrees of freedom	Sum of squares	Mean square	*F*	*P*
Rabbits (A)	2	45·7917	22·8959	4·43	>0·01, <0·05
Hyaluronidase (B)	1	736·3334	736·3334	142·52	<0·001*
Histamine (C)	1	720·7501	720·7501	139·50	<0·001*
Salicylate (D)	1	602·0834	602·0834	116·53	<0·001*
		Interactions			
A/B	2	11·7916	5·8958	1·14	>0·05
A/C	2	22·8749	11·4375	2·21	>0·05
A/D	2	4·2916	2·1458	—	—
B/C	1	8·3332	8·3332	1·61	>0·05
B/D	1	26·9999	26·9999	5·23	>0·01, <0·05
C/D	1	720·7499	720·7499	139·50	<0·001*
A/B/C	2	3·0418	1·5209	—	—
A/B/D	2	2·6251	1·3126	—	—
A/C/D	2	7·8751	3·9376	—	—
B/C/D	1	8·3335	8·3335	1·61	>0·05
A/B/C/D	2	10·0415	5·0208	—	—
Error	24	124·0000	5·1667	—	—
Total	47	3055·9167	—	—	—

* Highly significant.

reference to Table 6, salicylate in the dosage used completely inhibited the capillary-damaging action of histamine diphosphate injected in 0·01 mg. amounts. The skin-diffusing action, moreover, of a hyaluronidase-histamine mixture, was reduced, by means of salicylate, to that of the hyaluronidase component alone.

DISCUSSION

Allusion has already been made to the discrepancy between the *in vivo* inhibitory effect of salicylate on the skin-diffusing activity of hyaluronidase described by Guerra (1946*a*, *b*) and the failure of *in vitro* inhibition of viscosity-reducing activity reported by Swyer (1948), as well as to the tentative explanation offered. The evidence presented in this paper regarding the potentiation of hyaluronidase skin-diffusing activity by histamine and its inhibition by salicylate lends substantial support to such an explanation. At the same time, the effect of salicylate in preventing increase of capillary permeability by histamine is in itself a finding of considerable interest.

If, as seems to be clear, the anti-rheumatic action of salicylates is not due, as Guerra proposed, to their alleged inhibitory effect on hyaluronidase, the question naturally arises as to whether it can be ascribed to their anti-histamine properties. This problem is briefly discussed by Swyer (1948), but the conclusion is reached that the evidence available in the literature on the effects of salicylate on immunological reactions, though fairly extensive, is scarcely sufficient to afford a solution. At the same time, however, such an interesting possibility cannot be excluded.

SUMMARY

1. Using factorial tests on rabbits, comparisons have been made of the skin-diffusing activities of hyaluronidase prepared from bull semen and rattlesnake venom, and their effects in increasing capillary permeability as evidenced by the pontamine blue reaction, both before and after the administration of

sodium salicylate. In the same way, the effect of salicylate on the skin-diffusing and capillary-damaging actions of hyaluronidase, histamine and a hyaluronidase-histamine mixture have been studied.

2. The hyaluronidase preparation had no significant effect on capillary permeability, and its spreading action was not affected by salicylate. Snake venom, on the other hand, had a marked capillary-damaging effect which, along with its spreading action, was significantly reduced by salicylate. Histamine increased the spreading action of hyaluronidase in a strictly additive fashion, and its capillary-damaging action was inhibited by salicylate to a highly significant extent; salicylate reduced the spreading action of a hyaluronidase-histamine mixture to that of the hyaluronidase component alone.

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Failure of *in vitro* Inhibition of Hyaluronidase by Salicylates

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Guerra (1946 *a*, *b*) claimed that sodium salicylate in doses of 0·07 and 0·10 g./kg. caused 57 and 66 % reduction of the skin diffusion effect of hyaluronidase in rabbits. Moreover, he reported that in humans who had active rheumatic fever, or had suffered it, intradermal injections of hyaluronidase with Evans blue gave 'unique reactions with enormous diffusion of the dye and local oedema that sometimes occupies the arm injected with hyaluronidase'. Salicylates in these cases inhibited the enzyme and reduced its spreading effect in connective tissues. On the basis of these findings he believed that hyaluronidase played an important part in the pathogenesis of rheumatic fever and that the anti-rheumatic action of salicylates could be explained by their inhibition of the enzyme.

Since considerable and increasing attention is being paid to these reports, it seems proper to record the results of some experiments on the *in vitro* effect of salicylates on hyaluronidase action, in which, using a viscosimetric technique, the accuracy of which is far greater than that employing skin diffusion, no direct inhibitory effect was observed except with relatively enormous salicylate concentrations. At the same time, the action of salicylates will be compared with that of heparin, a true hyaluronidase inhibitor (McClean, 1942; Rogers, 1946).

METHODS

The effects of sodium salicylate, acetylsalicylic acid and heparin on the activity of hyaluronidase in decreasing the viscosity of buffered solutions of potassium hyaluronate were studied by a modification of the hyaluronidase assay technique described by Swyer & Emmens (1947). The enzyme preparation was the standard freeze-dried bull-semen filtrate described by those authors. Activity of the enzyme is expressed by the flow-time index, which has been shown to be proportional over a fairly wide range to the logarithm of the concentration of the enzyme. In all cases, the reaction mixture consisted of 0·2% potassium hyaluronate, 1 ml.; McIlvaine's citric acid phosphate buffer, M/60, pH 7·0, containing NaCl (3·5 g./l.), 0·5 ml.; appropriate dilution of the substance to be tested, 1 ml.; enzyme solution, 0·5 ml. The reaction took place, and the flow-time through the viscosimeter tube was read (20 min. after adding enzyme to substrate) at 34° (see Swyer & Emmens (1947) for details).

RESULTS

In the first test, serial dilutions of 1 % (w/v) sodium salicylate solution were tested against enzyme solutions containing 0·5 unit (Swyer & Emmens, 1947) and 0·125 unit/ml. The results, shown in Table 1, indicate that over this range of concentration of salicylate no inhibition occurs, while with 0·33 % salicylate in the reaction mixture there appeared to be facilitation.

Table 1. *Effect of sodium salicylate on hyaluronidase action*

Na salicylate in reaction mixture (%)	pH	Flow-time index (Hyaluronidase, unit/ml.) (0·125)	(0·25)	(0·5)	Notes
0	—	605	—	872	Control
0·003	—	677	—	834	No inhibition
0·033	—	624	—	860	
0·066	—	605	—	872	
0·16	—	644	—	872	
0·33	—	733	—	904	Facilitation
0	7·0	560	765	—	Control
0·33	7·0	666	890	—	Facilitation
0·833	7·0	653	835	—	
1·66	6·6	276	515	—	Inhibition
2·5	6·4	133	292	—	
3·33	6·2	150	318	—	

The tests were repeated with dilutions of 10% sodium salicylate, using enzyme solutions containing 0·25 and 0·125 unit/ml. The results (Table 1) confirm the facilitation effect over the range of salicylate concentrations 0·33–0·83% in the reaction mixture. When the salicylate concentration rose to 1·66% and higher, increasing inhibition was observed, but it was also found that the pH of the reaction mixture fell progressively with increasing salicylate concentration (see below).

Acetylsalicylic acid was first tested in serial dilutions of a 1% (w/v) solution against an enzyme solution containing 0·5 unit/ml. Complete inhibition occurred when the concentration in the reaction mixture reached 0·33%. Further tests with concentrations ranging between 0·066 and 0·33% against enzyme solutions containing 0·125 and 0·25 unit/ml. revealed increasing inhibition over this range (Table 2). The pH of the reaction mixtures in one of these latter tests was estimated with indicators using the British Drug Houses capillator outfit, and equivalent mixtures were made, using acetic acid in place of acetylsalicylic acid, so as to obtain as near as possible the same pH. The degree of enzyme activity in these mixtures resembled quite closely that found in the former ones, thus indicating that the observed inhibitory effect could be explained simply on the basis of hydrogen ion concentration (Table 2). As can be seen from Table 1, the fall of pH with strong sodium salicylate mixtures was considerably less marked than with acetylsalicylic acid; the greatly increased molarity of the mixture is probably to be held partly responsible for the inhibitory effect with strong sodium salicylate mixtures.

Table 2. *Effect of acetylsalicylic acid and acetic acid on hyaluronidase action*

(*a*) Addendum: acetylsalicylic acid

Acetylsalicylic acid in reaction mixture (%)	pH	Flow-time index (Hyaluronidase, 0·5 unit/ml.)	
0	—	872	
0·003	—	898	
0·033	—	840	
0·066	—	848	
0·16	—	645	
0·33	—	0*	
		(Hyaluronidase, 0·125 unit/ml.)	(Hyaluronidase, 0·25 unit/ml.)
0	7·0	560	775
0·066	6·6	433	545
0·133	5·4	388	297
0·2	5·0	174	166
0·266	4·2	66	157
0·33	3·8	0*	111

(*b*) Addendum: acetic acid

pH	(Hyaluronidase, 0·25 unit/ml.)
7·0	—
6·6	505
5·4	350
5·0	155
4·2	123
3·8	0*

* Complete inhibition.

The effect of heparin on hyaluronidase activity was investigated by using serial dilutions of a solution of heparin containing 10 mg. (101 Toronto units/mg.) in 10 ml. water in the same way as previously described, the hyaluronidase solution containing 0·25 unit/ml. From Table 3 it can be seen that inhibition is complete when the reaction mixture contains 6·6 units of heparin/ml. (i.e. 0·0066%), while below this concentration the degree of inhibition decreases until, with 0·163 unit/ml. in the reaction mixture, it disappears. In no case was the pH of the reaction mixture altered from its initial value of 7·0.

Table 3. *Effect of heparin on hyaluronidase action*

Heparin: (units/ml. in reaction mixture)	Flow-time index (Hyaluronidase, 0·25 unit/ml.)	Notes
0	775	Control
0·033	790	No inhibition
0·163	800	
0·33	405	Increasing inhibition
1·63	375	
3·3	152	
6·6	0	Complete inhibition

Table 4. *Combined effect of variation of heparin and hyaluronidase concentrations—factorial scheme showing viscosity index for each of sixteen combinations of hyaluronidase and heparin*

	Heparin (units/ml.)			
Hyaluronidase (unit/ml.)	40	20	10	5
0·125	0	0	0	96
0·25	0	0	72	134
0·5	0	85	102	420
1·0	195	273	495	745

Flow-time indices

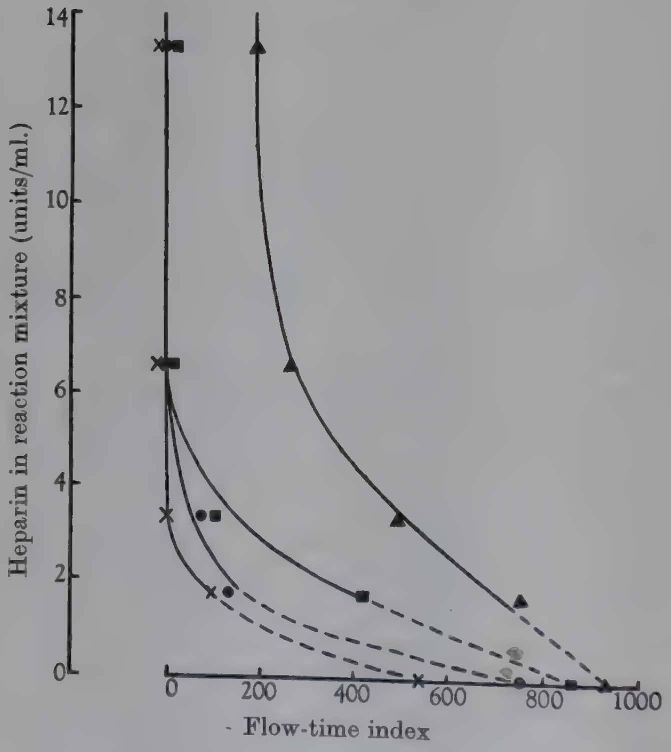

Fig. 1. Effect of concentration of heparin on the viscosity reducing activity of hyaluronidase solutions of different strengths: ×—× 0·125 unit/ml.; ●—● 0·25 unit/ml.; ■—■ 0·5 unit/ml.; ▲—▲ 1·0 unit/ml.

The effect of combined variation of heparin and of enzyme concentration was studied by means of a factorial test involving four concentrations of heparin and four of enzyme, the sixteen combinations being tested in random order (Table 4). The results are illustrated by Fig. 1, in which the flow-time index is plotted against concentration of heparin for the four concentrations of enzyme; it is clear that a relationship exists between quantity of heparin and amount of enzyme inhibited, whereas in the case of salicylates, the evidence suggested that no such relationship held.

DISCUSSION

Comparison of the effects of salicylates with those of heparin on the viscosity-reducing activity of hyaluronidase shows quite clearly the difference between a true hyaluronidase inhibitor, heparin, which, in a concentration of 0·0066 % in the reaction mixture, completely inhibits the activity of an enzyme solution containing 0·25 unit/ml. without altering the pH, and the salicylates, whose inhibitory action, manifested only in very high concentrations (more than 3 % for sodium salicylate and 0·33 % for acetylsalicylic acid), appears to be directly due to their effect on the pH of the reaction mixture, or, in the case of sodium salicylate, to the combined effect of lowered pH and increased salt concentration. Since, however, a close correlation has been shown to exist between the skin-diffusing and viscosity-reducing activities of hyaluronidases (McClean, 1943; Humphrey, 1943), it is surprising that sodium salicylate should possess the inhibitory effect on skin diffusion as described by Guerra. The recent work of Hechter (1946, 1947) may help to provide an explanation for this discrepancy. This investigator has shown very clearly that the skin-diffusing activity of hyaluronidase depends not only on the concentration of the enzyme, but also on the volume of solution injected, or on the interstitial fluid pressure; if the latter be maintained as a result of increased capillary permeability (such as is produced by histamine and inflammation-inducing agents) the extent of spread is considerably greater than when there is no accompanying capillary change. It is suggested, therefore, that the hyaluronidase preparation used by Guerra contained histamine, or some substance with a similar action, and that sodium salicylate, by inhibiting this substance, reduced the apparent spreading effect of hyaluronidase. The validity of such an explanation is rendered more probable by the observations (Swyer, 1948) that histamine enhances the spreading action of hyaluronidase, as could be predicted from the findings of Hechter (1946, 1947), and that sodium salicylate in the dose employed by Guerra (0·1 g./kg. intravenously in rabbits) inhibits the ability of histamine to increase capillary permeability and thereby reduces the spreading action of a hyaluronidase-histamine mixture.

In view of the evidence which has been presented that hyaluronidase is not inhibited by salicylates in therapeutic concentrations, it would seem that Guerra's explanation of the anti-rheumatic action of these drugs in terms of hyaluronidase inhibition must be abandoned. The question naturally arises as to whether or no their apparent anti-histamine action can be invoked in this connexion, as well as whether the effect of hyaluronidase on the skin of rheumatic fever patients described by Guerra and its diminution after administration of salicylate can be explained by the same mechanism. A voluminous literature exists regarding the effects of salicylates on immunological reactions, but the evidence, though some is suggestive, seems to be scarcely sufficient to provide a complete answer to these questions. The response to hyaluronidase described by Guerra in rheumatic fever, however, appears to be of the nature of a hypersensitivity reaction, such as was described by Jones & Mote (1934) as occurring in rheumatic fever patients following repeated injections of very small amounts of rabbit serum. It is true that only a single injection of hyaluronidase appeared to have been given (in Guerra's paper no details are provided, nor is any mention made of control tests in normal subjects), but since streptococci of the type believed to be responsible for predisposing to rheumatic fever are hyaluronidase producers, sensitivity to hyaluronidase might well be of general occurrence in rheumatic fever. If such be the case, inhibition of histamine, which is believed to play a part in such hypersensitivity reactions, would account for the reduced response to intradermal injections of hyaluronidase after treatment with salicylate.

SUMMARY

1. Investigation of the effects of sodium salicylate and acetylsalicylic acid on the viscosity-reducing activity of hyaluronidase has shown that they are inhibitory only in relatively enormous concentrations (more than 3 % for sodium salicylate and 0·33 % for acetylsalicylic acid).

2. Such inhibition is apparently due to lowering of pH and, in the case of sodium salicylate, to increased salt concentration.

3. Heparin, on the other hand, is completely inhibitory in a concentration of 0·0066 %.

4. The significance of these findings in relation to the claims made by Guerra (1946*a*) of the association of hyaluronidase with rheumatic fever, and the anti-rheumatic action of salicylates is discussed.

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The Influence *in vitro* of Deoxycorticosterone on Glycogen Formation in Muscle

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The main action of the adrenal cortical hormone is generally believed to be the regulation of glycogen metabolism. In a series of experiments from this Institute the phosphorylation of glycogen was investigated in normal and adrenalectomized animals. This reaction, by which glucose-1-phosphate is formed from glycogen, is the first phase in the breakdown of glycogen. It was shown that the phosphorylation of glycogen *in vitro* was greatly reduced in tissue from adrenalectomized animals (Verzár & Montigel, 1942; Doetsch, 1945).

In order to study directly the synthesis of glycogen, we chose the technique of Gemmill (1940, 1941) and Gemmill & Hamman (1941) by which the synthesis of glycogen from glucose can be observed in the surviving diaphragm of the rat. The rat's diaphragm is suitable for this experiment because, owing to its thin structure, it can be kept alive in Ringer solution for a few hours if saturated with oxygen. If glucose is added to this solution, the diaphragm synthesizes glycogen. Gemmill (Gemmill, 1940, 1941; Gemmill & Hamman, 1941) and also

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Hechter, Levine & Soskin (1941) have shown that this glycogen synthesis can be considerably increased by the addition of insulin. The diaphragm of adrenalectomized rats, too, synthesized glycogen from glucose, and insulin gave the same increase as in normal rats (Koepf, Horn, Gemmill & Thorn, 1941). It thus appeared that the main disturbance in glycogen metabolism in adrenalectomized animals is not in the synthesis of glycogen.

We therefore investigated the effect of the addition of deoxycorticosterone (DOC) on the glycogen formation in the diaphragm of normal and adrenalectomized rats. We found that DOC inhibits completely the formation of glycogen and also the effect of insulin on this formation.

METHODS

The method of Gemmill (1940, 1941) was used with slight modification, but concentrations of insulin and glucose were kept within physiological limits. Rats of 100–150 g. body weight of both sexes and of our own breed were killed by a blow on the head and decapitation and were bled as much as possible. The diaphragm was rapidly dissected out and cut into three parts of almost equal weight (from 100 to 150 mg.). The posterior thicker part of the diaphragm was immediately weighed on a torsion balance, dropped into 20% (w/v) NaOH (1 ml.) and hydrolyzed on a boiling water bath for the estimation of the initial glycogen content. The two remaining pieces of diaphragm were also weighed immediately, washed in phosphate buffer Ringer solution and dropped into 50 ml. Erlenmeyer flasks containing 10 ml. of the phosphate buffer Ringer solution, which was prepared according to Hastings, Muus & Bessey (1939) and contained 8 g. NaCl, 0·2 g. KCl, 0·2 g. $CaCl_2$, 0·1 g. $MgCl_2$, 0·8 g. $Na_2HPO_4 . 2H_2O$ and 0·15 g. KH_2PO_4/l. (pH *c.* 7·2–7·4 as measured by Bayer pH papers nos. 7 and 8). The following substances were then added to the Ringer solution: 100 mg. glucose/100 ml., 1 unit insulin/100 ml., 0·01–10 mg. DOC/100 g. and 0·25–0·50% ethanol. A solution of insulin (Eli Lilly) with an activity of 40 units/ml. was diluted with Ringer solution to a concentration of 1 unit/ml. Usually 0·1 ml. of this solution was dropped into the flasks containing 10 ml. of the phosphate buffer Ringer solution. The final insulin concentration was therefore 1 unit/100 ml. DOC (100 mg.) was dissolved in 5 ml. ethanol and 5 ml. water were added; 0·1 ml. of this solution was added to the flasks if a concentration of 10 mg./100 ml. was required. According to Miescher & Meystre (1943) DOC has a solubility of 0·012% in water and so all the DOC in our experiments was dissolved by the Ringer solution. Solutions of 0·1 and 0·01% DOC were used if lower concentrations were needed. If diaphragm is permeable to insulin and DOC, the hormone concentrations in the muscle and in the Ringer solution should be identical. One of the lateral pieces of diaphragm was always kept as a control without the addition of the hormone. The flasks containing the pieces of diaphragm were at once filled with oxygen, closed with rubber stoppers and shaken in a water bath at 37°. From time of death to the beginning of shaking only 4–5 min. elapsed. It was therefore not necessary to prepare the diaphragms at 0°. In the majority of experiments the shaking in the water bath was continued for 1·5 hr., and the pieces of diaphragm were then dropped into 1 ml. of 20% (w/v) NaOH and hydrolyzed on a boiling water bath until the solution was clear (10–15 min.). The glycogen was then precipitated from the hot solution with 2 ml. of ethanol and the precipitation completed by standing in ice water for 0·5 hr. The glycogen was centrifuged off, the supernatant poured away, the glycogen stirred up with 2 ml. of ethanol, again centrifuged at 3000 r.p.m. for 3–5 min. and dried on a boiling water-bath. According to the amount of precipitate 1–2 ml. $2\text{N-}H_2SO_4$ were added and the glycogen hydrolyzed on a boiling water bath for 1·5 hr. The hydrolysate was washed into a 50 ml. measuring flask and made alkaline with 2·3 ml. of $2\text{N-}Na_2CO_3$. Depending on the amount of glycogen 2–10 ml. were taken from the flask and the sugar content estimated by the Hagedorn-Jensen method, according to Hallmann (1939). The glycogen contents are given as mg. glycogen/100 g. wet muscle.

RESULTS

The results are reported in Tables 1 and 2. Only the mean values of results obtained with several animals are given. The glycogen contents are given at the beginning of the experiment (initial glycogen content) and after 1·5 hr. with additions of the different substances as glucose, insulin, DOC and ethanol. The tables show the differences between the glycogen contents before and after incubation with a given addition. More important, however, are the differences between the results with different additions, i.e. glucose and glucose + insulin, or no glucose and glucose + insulin, or those with DOC, which give the true effect of the addition. From Tables 1 and 2 these values can easily be calculated. Since all values are arithmetic mean values the standard error for the differences in each series has been calculated. One example of a series with 10 single experiments is given in Table 2. Here the differences between the results with different additions are also shown.

1. *Action of glucose*

The experiments on 25 animals (Table 1, series 1) show that the glycogen content of the diaphragm in phosphate buffer Ringer solution without glucose decreased in 1·5 hr. by a mean value of 57 mg./100 g. Addition of glucose (100 mg./100 ml.) (without insulin) produced glycogen in normal rats (series 2) and also in rats which had been starved for 2 days (series 6). In the former series the glycogen content increased by 46 mg./100 g. compared with the initial value and in the latter by 126 mg./100 g. If these values are compared with those of series 1 (without glucose), where the glycogen content decreased by 57 mg./100 g., the total glycogen production can be calculated to be 103 and 183 mg./100 g. respectively. In single experiments differences of 50 mg./100 g. are not regarded as real, but in a series of several experiments the mean error becomes much smaller, as is shown in the tables.

Table 1. *Changes in the glycogen contents of diaphragms of normal, starved and adrenalectomized rats on incubation with and without the addition of glucose, insulin and deoxycorticosterone*

Series no.	No. of rats	Mean initial glycogen content (mg./100 g. of muscle)	Additions to suspending fluid				Mean glycogen content after 1·5 hr. (mg./100 g. of muscle)	Mean differences (mg./100 g. of muscle)	S.E. of mean differences
			Glucose (mg./100 ml.)	Insulin (units/100 ml.)	DOC (mg./100 ml.)	Ethanol (%)			
1	25	239	—	—	—	—	182	− 57	12
2	60	280	100	—	—	—	326	+ 46	13
3	13	296	100	0·1	—	—	397	+101	20
4	14	265	100	0·5	—	—	494	+229	17
5	102	233	100	1	—	—	363	+130	8
6	13*	100	100	—	—	—	226	+126	8
7	13*	100	100	0·5	—	—	422	+322	19
8	8	170	—	0·5	—	—	136	− 34	23
9	11	165	—	1	—	—	142	− 23	9
10	8	129	100	100	—	—	279	+150	17
11	8	270	100	—	10	0·50	185	− 85	22
12	14	305	100	—	5	0·25	298	− 7	14
13	12	301	100	—	1	0·50	297	− 4	16
14	11	261	—	—	5	0·25	203	− 58	13
15	14	326	100	—	—	0·50	377	+ 51	20
16	10	175	100	1	—	0·50	321	+146	24
17	12	187	100	—	10	—	155	− 32	14
18	12	277	100	1	10	—	245	− 32	24
19	13	157	100	1	10	0·50	88	− 69	17
20	30	246	100	1	5	0·25	244	− 2	17
21	20	321	100	1	1	0·50	470	+149	20
22	6	203	100	1	0·5	0·25	318	+115	31
23	15	183	100	1	0·1	0·50	291	+108	28
24	6	131	100	1	0·05	0·25	290	+159	25
25	12	265	100	1	0·01	0·25	399	+134	25
26*a*	7	297	100	1	10	0·50	166	−131	39
26*b*	7	297	100	100	10	0·50	191	−106	32
27*a*	23†	225	100	—	—	—	288	+ 63	18
27*b*	23†	225	100	0·5	—	—	407	+182	15
28*a*	16†	164	100	1	—	—	282	+118	20
28*b*	16†	164	100	1	10	0·50	106	− 58	12

* After a starvation period of 2 days.

† Adrenalectomized animals.

2. *Action of insulin on glycogen production*

In Table 1, series 3, 4 and 5 show the effect on glycogen formation of 0·1, 0·5 and 1 unit of insulin/100 ml. respectively. To 10 ml. phosphate buffer Ringer solution in which was suspended one third of a diaphragm 0·1 unit of insulin was added (series 5). This corresponds to an insulin content of 1 unit/100 ml. of Ringer solution, or 100 g. of muscle. Insulin already at a dose of 0·1 unit/100 ml. increases glycogen production by about 50 mg./100 g. (series 3) as compared with glucose alone (series 2). With 0·5 unit the insulin effect is almost maximal giving an increase of 183 mg./100 g. as compared with glucose alone (series 4). In series 5, 102 experiments with insulin (1 unit/100 ml.) are collected. The mean value for the glycogen production is 130 mg./100 g. The values of this series are also plotted against the number of animals in Fig. 1. In this figure the total number of experiments in which a given increase (± 20 mg./100 g.) in glycogen content

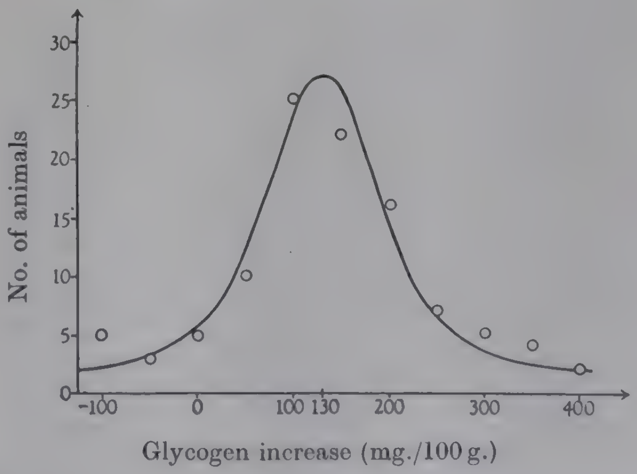

Fig. 1. Glycogen production from glucose (100 mg./100 ml.) by diaphragms of normal rats with insulin (1 unit/100 ml.) in 102 experiments. The ordinate value of each point indicates the number of diaphragms which produced the amount of glycogen (±20 mg./100 g. muscle) given by the abscissa. The solid line is the normal probability equation.

was obtained is plotted, as the ordinate, against the magnitude of the given increase (abscissa).

Nearly 80 % of all experiments fall inside the values of + 50 to + 300 mg./100 g. There are, however, even in this series a certain number of negative results which are included in the mean value decreasing this to 130 mg./100 g. This seems to be lower than the mean value with 0·5 unit insulin/100 ml. in a series with only 14 experiments, but the difference is not significant.

In the experiments of Gemmill and co-workers (Gemmill, 1940, 1941; Gemmill & Hamman, 1941; and Koepf *et al.* 1941), 100–200 units insulin/100 ml. were used, but these authors observed about the same maximal increase of glycogen production as we found with much smaller quantities of insulin. We have therefore compared the action of different quantities of insulin on two parts of the same diaphragm. (The third part of the diaphragm was always used to obtain the initial glycogen value.)

In a series of 4 experiments we found glycogen productions, with 0·5 unit insulin/100 ml., of 65 mg./100 g. and, with 1 unit/100 ml. of 70 mg./100 g. In another series of 8 experiments we found glycogen productions, with 1 unit/100 ml. of 136 mg./100 g. and, with 100 units/100 ml. of 150 mg./100 g. (series 10).

Thus, increasing the concentration of insulin above 0·5 unit/100 ml. has very little effect, and on the same diaphragm 0·5 unit acts like 1, or 1 like 100 units/100 ml. It must be remembered however that the activity of different doses of insulin cannot be compared on different diaphragms. The largest values for glycogen content, above 400 mg./100 g., were found with 0·5 unit/100 ml. in series 4 and 7, and these are not less than with 100 units/100 ml. in series 10.

We have also measured the time in which insulin has this effect. Insulin (1 unit/100 ml.) was added to both parts of the diaphragm and the incubation was stopped at different times. Glycogen production is especially active in the first 0·5 hr. being about 100 mg./100 g. After that, only about 10–20 mg./100 g. glycogen is formed in each 0·5 hr.

Control experiments were also done to see whether insulin has any action if it is added without glucose. Series 8 and 9 show that no glycogen was formed and the initial glycogen was broken down as in series 1 where neither glucose nor insulin had been added. Thus insulin cannot inhibit glycogenolysis.

Mention must be made of series 6 and 7 in which animals starved for 2 days were used. Such control experiments were necessary, since results obtained with adrenalectomized rats might be ascribed to the fact that they were starving. The glycogen content of the muscles in these animals was very low, about 100 mg./100 g. The glycogen content after addition of glucose alone was 226 mg./100 g., with glucose and insulin (0·5 unit/100 ml.) 422 mg./100 g. The insulin effect is 196 mg./100 g. (difference: 322 − 126 = 196) and is equal to that of animals on a normal diet (see series 2 and 4, difference: 229 − 46 = 183).

3. *Action of deoxycorticosterone*

We tested next the action of DOC with and without glucose and with and without insulin. The results are shown in series 11–18 (Table 1). In series 11, 12 and 13 the action of 10, 5 and 1 mg. DOC/100 ml. of solution (or g. of muscle) in the presence of glucose is shown. In all experiments no glycogen was produced from glucose. In series 11 there was even a breakdown of 85 mg./100 g. as compared with the initial glycogen content. If we compare series 2, in which a glycogen production of 46 mg./100 g. from glucose was demonstrated, the real decrease in glycogen in series 11 is 131 mg./100 g. In series 12 and 13 with 5 and 1 mg. DOC/100 ml. only the glucose effect was abolished by DOC. In series 14 no glucose but 5 mg.DOC/100 ml. were added and a decrease of glycogen of 58 mg./100 g. resulted, which is very similar to the decrease without any addition (series 1).

After addition of DOC the Ringer solution contains 0·25–0·50 % ethanol, as pointed out in the Methods section. Series 15 and 16 show that ethanol without DOC has no action whatever on the glycogen formation with glucose and with glucose + insulin. The results are the same as in series 2 and 5.

Experiments with ethanol (1 %), glucose (100 mg./100 ml.) and insulin (1 unit/100 ml.) showed a diminished glycogen production (by about 30 mg./100 g.) in comparison with glucose and insulin alone; 0·5 % ethanol, the concentration used in our previous experiments with DOC, had no action. Nevertheless we now tested whether DOC has an inhibitory action on glycogen production in aqueous solution, i.e. without ethanol. It is possible to dissolve about 0·01 % DOC in water (Miescher & Meystre, 1943). We took 100 mg. of finely pulverized crystalline DOC and shook it for 3 hr. in 500 ml. of phosphate buffer Ringer solution, which thus became saturated with DOC. After addition of 100 mg. glucose/100 ml. we dropped the diaphragms into 10 ml. of this solution and performed the experiment in the usual manner. The results are shown in series 17. With glucose and without insulin, DOC caused a glycogen breakdown of 32 mg./100 g., or 78 mg./100 g. when compared with series 2. The inhibition of glycogen production and the increase of glycogen breakdown by DOC is of the same order as that caused by DOC with 0·5 % ethanol (series 11 and 19). Thus ethanol in the concentrations used by us does not influence the inhibitory action of DOC on glycogen production.

All these experiments show that DOC does not increase glycogen formation in muscle, but rather inhibits glycogen production from glucose.

4. *Combined action of deoxycorticosterone and insulin*

Since the preliminary experiments of sections 1–3 have shown the smallest effective doses of insulin on glycogen formation, and of DOC on glycogen breakdown, we can describe our main experiment, i.e. the action of DOC in the presence of insulin.

In Table 1 these results are shown as mean values in series 19–26*b*. From series 19 it is obvious that, in the presence of 100 mg. glucose/100 ml. and 1 unit insulin/100 ml. with 10 mg. DOC/100 ml., no glycogen is formed but as much is broken down as if no glucose and no insulin were present. In series 20 with 5 mg. DOC/100 ml., less action is seen; there is no production of glycogen but also no decrease. In series 21 with 1 mg. DOC/100 ml., there is an increase of glycogen content, i.e. the action of insulin is not antagonized. Smaller DOC concentrations of 0·5–0·01 mg./100 ml. in the series 22–25 are equally without antagonistic action. In these the results are the same as with insulin alone; about 100–150 mg. glycogen/100 g. are formed. In all the series a corresponding amount of ethanol was added to the controls without DOC. In all cases ethanol in concentrations of 0·25 and 0·50 % was quite without influence on glycogen synthesis and breakdown.

In series 18 an aqueous solution of DOC without ethanol was used, parallel to series 17 mentioned above. Glycogen was broken down at the same rate as in series 19.

Series 19–21 contain the main results of this paper. We give therefore 10 consecutive single experiments from series 19 in Table 2, as an example of each series with its controls.

From these single experiments the same follows as from the mean values of Table 1. In the last column the total breakdown of glycogen is calculated by comparing the two glycogen contents after 1·5 hr., those with glucose and insulin alone and those with additional DOC. With 10 mg. DOC/100 ml. the effect on glycogenolysis was 174 mg./100 g. as mean value of these 10 experiments. In Table 1 comparing series 5 and 19 we calculate a DOC effect on glycogen breakdown of 199 mg./100 g. The single differences in each of the columns 2–1 and 3–1 vary considerably. Nevertheless except with the second experiment all differences in column 3–2 are very similar.

The effect of DOC on glycogen breakdown is not a direct antagonistic action against added insulin, as is shown by a comparison with series 11 where glycogen is broken down with DOC without insulin, as well as with DOC and insulin in series 19.

The series 26*a* and 26*b* prove the same. There is no correlation between the doses of insulin and DOC, because 1 unit insulin/100 ml. as well as 100 units/100 ml. is inhibited by 10 mg. DOC/100 ml.

5. *Action of insulin and DOC on the diaphragm of adrenalectomized adynamic rats*

The action of DOC was next studied with muscle from adrenalectomized animals.

Male rats, of 100–150 g. body weight, of the Institute's own breed, were adrenalectomized. They recovered from the operation in 3 days and increased in weight. The animals were killed between the 5th and 10th day if a decrease in weight and adynamia had appeared.

Series 27*a*–28*b* (Table 1) summarize the experiments by showing mean values. Series 27*b* and 28*a* confirm what was already known from Gemmill's experiments (Koepf *et al.* 1941), that the diaphragm of adrenalectomized rats *in vitro* produces glycogen in the same way as that of normal animals. If glucose only was present (series 27*a*), 63 mg. glycogen/100 g. were formed. With 0·5 unit insulin/100 ml. (series 27*b*) 182 mg./100 g. and with 1 unit/100 ml. (series 28*a*) 118 mg. glycogen/100 g. were produced.

Table 2. *Typical experiments demonstrating the action of deoxycorticosterone (10 mg./100 ml.) on glycogen formation by diaphragms from glucose and insulin*

Initial glycogen content (mg./100 g.) 1	Glycogen content after 1·5 hr. (mg./100 g.): With glucose and insulin 2	Glycogen content after 1·5 hr. (mg./100 g.): With glucose, insulin and DOC 3	Differences in glycogen content (mg./100 g.): 2–1	Differences in glycogen content (mg./100 g.): 3–1	Differences in glycogen content (mg./100 g.): 3–2
160	240	40	+ 80	−120	−200
140	40	40	−100	−100	0
80	230	40	+150	− 40	−190
370	370	160	0	−210	−210
180	360	110	+180	− 70	−250
40	250	70	+210	+ 30	−180
160	290	80	+130	− 80	−210
180	360	110	+180	− 70	−250
90	200	80	+110	− 10	−120
160	290	160	+130	0	−130
Means: **156**	**263**	**89**	**+107**	**− 67**	**−174**

If, however, in series 28*b* to 1 unit insulin/100 ml. 10 mg. DOC/100 g. were added, the glycogen content decreased by 58 mg./100 g. Thus the results were exactly the same as in normal animals in the series 2, 4, 5, 18 and 19.

6. *Action of insulin and DOC on glycogen formation from glucose-1-phosphate*

The experiments on glycogen formation by normal diaphragms were repeated using, instead of glucose, glucose-1-phosphate (Cori ester) as substrate. This substance, according to present knowledge, is the first product of glycogen breakdown, and possibly also the last intermediate in glycogen synthesis. Cori, Schmidt & Cori (1939) and others have shown that *in vitro* an enzyme fraction of muscle extracts, the so-called phosphorylase, can synthesize glycogen from glucose-1-phosphate.

The glucose-1-phosphate which was used for these experiments was prepared according to Cori, Colowick & Cori (1937). For these experiments the concentration used was equivalent to 100 mg. glucose/100 ml. (150 mg. glucose-1-phosphate/100 ml.). This was verified after hydrolysis by estimation of glucose by the Hagedorn-Jensen method.

Table 3. *Glycogen content of diaphragm from normal animals after incubation with glucose-1-phosphate*

Series no.	No. of rats	Mean initial glycogen content (mg./100 g. of muscle)	Additions to suspending fluid: Glucose-1-phosphate (mg./100 ml.)	Insulin (units/100 ml.)	DOC (mg./100 ml.)	Ethanol (%)	Mean glycogen content after 1·5 hr. (mg./100 g. of muscle)	Mean differences (mg./100 g. of muscle)	S.E. of mean differences
29	16	329	150	—	—	—	282	− 47	25
30	8	231	150	1	—	—	225	− 6	27
31	8	425	150	—	10	0·50	285	−140	53

The results are shown in Table 3. In series 29 (with glucose-1-phosphate alone) the glycogen decreased in the same way as in series 1 (Table 1) without glucose. Even with 1 unit insulin/100 ml. added there was no glycogen synthesis. DOC (5 mg./100 ml.) increased the glycogenolysis, as series 31 shows.

Thus glucose-1-phosphate cannot act as substrate for glycogen formation with intact muscle. Since Verzár & Wenner (1948) have found that minced muscle can synthesize glycogen from glucose-1-phosphate, the explanation seems to be that glucose-1-phosphate cannot diffuse into the intact muscle and is therefore not used. This is the more probable since it is known that glucose phosphates are only formed inside the cells and do not diffuse in or out of them.

DISCUSSION

We have thus confirmed the finding of Gemmill (Gemmill, 1940, 1941; Gemmill & Hamman, 1941) and of Soskin (Hechter *et al.* 1941) that the surviving diaphragm of rats can produce glycogen from glucose in the presence of insulin. We were also able to show that, contrary to the above authors, even without insulin glycogen can be formed in a solution containing not more than 100 mg. glucose/100 ml. Insulin increases this glycogen production three or four times. Whilst the American authors used 100–200 units insulin/100 ml., we found that even with 0·5–1 unit/100 ml. an almost maximal glycogen production was obtained. The glycogen production occurs mainly in the first half hour and slows down afterwards.

Diaphragms of adrenalectomized rats produced glycogen in the same amounts as those of normal animals. Thus the specific disturbance in adrenal cortical insufficiency cannot be an inability to produce muscle glycogen from glucose.

It was also shown that the addition of DOC with glucose alone, or with glucose and insulin, totally inhibited glycogen formation. The hormone was active in quantities of 1–10 mg./100 g. muscle, or for a diaphragm of 100 mg. in quantities of 1–10 μg. About 1 mg. DOC daily would be needed to keep alive an adrenalectomized rat of 150 g. body weight with 50 g. muscle tissue; this corresponds to about 2 mg./100 g. muscle. Thus the concentrations which were effective in these experiments *in vitro* are equivalent to the doses needed to keep an adrenalectomized animal alive.

In these experiments, therefore, DOC acts by increasing the breakdown of glycogen (glycogenolysis). This is in accordance with the fact previously announced in several papers from this laboratory (Verzár & Montigel, 1942, 1943*a*, 1943*b*; Doetsch, 1945), that after adrenalectomy the phosphorylation of glycogen by muscle (and liver) decreases. Glycogen-phosphorolysis is the first step in glycogen breakdown to form glucose-1-phosphate. As was shown by the above authors, DOC acts by accelerating this process.

Since however it is known that the same enzyme (phosphorylase) can also catalyze the synthesis of glycogen (Cori *et al.* 1939; Cori, Green & Cori, 1942; Kiessling, 1939; Ostern & Holmes, 1939), it has been thought until now that the disturbance might be on the side of glycogenesis rather than of glycogenolysis. The experiments in this paper show that the disturbance in the undamaged muscle lies only on the side of glycogenolysis, which is greatly increased by DOC in normal as well as in adrenalectomized animals.

It is difficult to explain the decrease of glycogen in the organs of adrenalectomized animals. The apparent result of the administration of DOC or other adrenal cortical hormones *in vivo* is an increase of glycogen content, as we ourselves and others have shown (e.g. Verzár & Montigel, 1943*a*, 1943*b*); Koepf *et al.* (1941, 1942) have also shown that in liver slices of adrenalectomized animals which were starved for 24 hr. the so-called total carbohydrate was lower than in normals; and that normal and adrenalectomized animals produced more total carbohydrate from lactate and pyruvate if cortin was previously injected.

In spite of these difficulties, our experiments can only be explained by assuming that DOC increases the glycogenolysis and thus counteracts the effect of insulin, which increases glycogenogenesis.

Since it was shown by Verzár & Wenner (1948), that the minced muscle of adrenalectomized animals can produce glycogen from Cori ester, while the undamaged diaphragm cannot, it seems that glucose-1-phosphate cannot diffuse into the undamaged muscle fibres (Conway, 1942). On the other hand, it can be used for glycogen formation in minced muscle from adrenalectomized as well as from normal animals. Thus the disturbance which leads to a decrease of phosphorylation of glycogen after adrenalectomy is on the breakdown side of glycogen metabolism: it is the first step of glycogen breakdown (glycogenolysis), the phosphorylation of glycogen in muscle, which is decreased after adrenalectomy. This process is increased in the whole muscle by DOC in normal and adrenalectomized animals. The increase of glycogenolysis through DOC can totally antagonize the action of insulin upon glycogen synthesis in muscle.

Further experiments with other steroids which we propose to do, are intended to show whether this effect which we have found is specific for DOC.

SUMMARY

1. The surviving diaphragm of the rat, in Ringer solution saturated with oxygen and buffered with phosphate, synthesizes glycogen when 100 mg. glucose/100 ml. are added. In pure Ringer solution, it breaks down its own glycogen stores.

2. The addition of 0·5–1 unit of insulin/100 ml. to the Ringer solution containing 100 mg. glucose/100 ml., increases the formation of glycogen. A further addition of insulin, up to 100 units/100 ml., produces no further increase in glycogen formation.

3. After the addition of 5–10 mg. of DOC/100 ml. to the Ringer solution there is a breakdown of glycogen in the diaphragm. When the Ringer solution contains glucose, the synthesis of glycogen is completely inhibited by the addition of even 1 mg. DOC/100 ml. When 1 unit of insulin/100 ml. is added to the Ringer solution with glucose, then 5–10 mg. DOC/100 ml. completely prevent the synthesis of glycogen. With 1 mg. DOC/100 ml. scarcely any action could be detected. Even when 100 units of insulin/100 ml. are added, no synthesis occurs in the presence of 10 mg. DOC/100 ml.

4. The muscles of adrenalectomized animals synthesize glycogen to the same extent as those of normal animals, and in these animals also DOC favours the breakdown of glycogen and inhibits the action of insulin.

5. If glucose-1-phosphate is taken instead of glucose then no synthesis of glycogen occurs even in the presence of insulin. The explanation may be that the cell membrane is non-permeable to this ester.

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Glycogen Production from Glucose-1-phosphate by Liver and Muscle of Normal and Adrenalectomized Animals

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It has been shown by Schumann (1940), Verzár & Montigel (1941, 1942, 1943*a*, 1943*b*) and by Kutscher & Wüst (1941, 1942) that, after adrenalectomy, phosphorylation of glycogen by muscle and liver (Doetsch, 1945) is decreased. Since some authors were not able to confirm this observation (Riesser, 1943–5; Smits, 1943–5; Helve, 1940) the experiments were repeated in this laboratory by Staehelin & Voegtli (unpublished) and the earlier results were fully confirmed. The first step in the breakdown of glycogen is the formation of glucose-1-phosphate and, as shown by Cori and his co-workers (Cori, Cori & Hegnauer, 1937; Cori, Colowick & Cori, 1937; Cori, Cori & Schmidt, 1939; Cori, Schmidt & Cori, 1939; Cori & Cori, 1939; Cori, Green & Cori, 1942), this reaction is reversible. The equilibrium mixture at pH 7 consists of 77 % glycogen and 23 % glucose-1-phosphate (Colowick & Sutherland, 1942). Glycogen has been produced *in vitro* with minced tissues (Kiessling, 1936, 1937; Ostern, Herbert & Holmes, 1939; Ostern & Holmes, 1939), tissue extracts (Cori, Cori & Schmidt, 1939; Cori & Cori, 1939; Mirski & Wertheimer, 1942) and with purified or even crystalline enzymes (Cori *et al.* 1937; Cori, Colowick & Cori, 1938; Cori *et al.* 1942; Kiessling, 1939*a*, 1939*b*; Colowick & Sutherland, 1942; Schäffner & Specht, 1938, 1939).

In our former experiments only the breakdown of glycogen was studied. It was therefore of interest to see whether the reverse reaction, the production of glycogen from glucose-1-phosphate, was influenced by adrenalectomy. New experiments of Koepf, Horn, Gemmill & Thorn (1941) and of Verzár & Wenner (1948) had shown that whole muscle of adrenalectomized animals is able to produce glycogen. We have therefore studied the problem as to whether muscle and liver of adrenalectomized animals can produce glycogen from glucose-1-phosphate.

METHODS

White rats of 150–300 g. of the laboratory stock were used. Adrenalectomy was carried out on males only. The animals appeared to be well again 2–3 days after the operation and became adynamic after 5–20, days when they were used for the experiments. Our test for adynamia was as follows: the animal was put on an almost vertical wire netting and its movements were observed. Normal animals always climb upwards, whilst adynamic animals cling on to the netting and fall down after a few minutes. We generally used animals which were able to keep up for at least 0·5 min.

The animals were pithed and then bled. The liver was immediately removed and pressed through a sieve. Muscles of the abdominal wall were cut up in a Latapie mincer. The methods of Ostern *et al.* (1939) and of Ostern & Holmes (1939) were used for the estimation of glycogen production in muscle and liver. Quantities of about 0·5 g. of tissue were quickly weighed on a torsion balance and then put in either (*a*) 1 ml. of 0·8 % NaCl containing 0·005 M-NaF, or (*b*) 1 ml. of a solution containing 0·005 M-NaF and glucose-1-phosphate of a concentration which was equivalent to a glucose content of 10 mg./ml. In certain cases 5 mg. of glycogen (Hofmann-La Roche), dissolved in 0·2 ml. of water, were added to the mixture (i.e. 1 g./100 g. muscle or liver). The muscle or liver mixture was kept at 20° and the glycogen content was measured after 0, 7, 15 and 30 min. Glycogen production usually reached its maximum after 15–30 min. After 1 hr. the newly formed glycogen had disappeared again (Ostern *et al.* 1939; Ostern & Holmes, 1939).

For glycogen estimation (Pflüger, 1909; Simonovits, 1933; Good, Kramer & Somogyi, 1933; Bomskov & Kaulla, 1942; Kiessling, 1939*a*; Cori & Cori, 1939, 1940) 2 ml. of 40 % (w/v) NaOH were added and the mixture was hydrolyzed on a boiling water bath for 15–30 min. The solution was made up with water to 5 ml. and 5 ml. of 96 % (v/v) ethanol were added. The mixture was kept at 0° for 30 min. and the precipitated glycogen was then centrifuged (3000 r.p.m.) for 5 min. Remaining glucose-1-phosphate was extracted by dissolving the precipitate in 1 % NaCl (2 ml.), reprecipitating the glycogen with ethanol (2 ml.) at 0° and centrifuging. The glycogen was finally dried by adding 96 % ethanol (2 ml.), centrifuging and evaporating the ethanol on a water bath. The glycogen was hydrolyzed with 2 N-H_2SO_4 for 2 hr. on a boiling water bath, the solution was transferred to a 50 ml. flask, made alkaline by addition of 2 N-Na_2CO_3 and diluted with water. The glucose was determined by the Hagedorn-Jensen method.

Glucose-1-phosphate was synthesized from glucose according to Cori *et al.* (1937). The preparation contained 80 % of the Ba salt. For the conversion to the Na salt, Ba was precipitated by Na_2SO_4 from a solution acidified with H_2SO_4 and the solution neutralized with 2 N-Na_2CO_3 (litmus).

RESULTS

The glycogen content of minced liver decreases after the death of the animal. To allow for this glycogenolysis, a control in which no glucose-1-phosphate was added was carried out for each experiment. Uncut

Table 1. *Glycogen production by minced normal liver*

			Glycogen content (mg./100 g.)			
Exp. no.	Wt. of rat (g.)	Time (min.)	Without additions	With addition of glucose-1-phosphate	Glycogen produced (mg./100 g.)	Remarks
4	230 ♂	0	4350	—	—	Concentration of glucose-1-phosphate 5%
		15	4300	5000	700	
		30	3890	4750	860	
5	215 ♀	0	6500	—	—	—
		15	6000	6700	700	
		30	5800	6900	1100	
13	250 ♂	0	6190	—	—	—
		7	6010	6440	430	
		15	5870	6470	600	
		30	5730	6020	290	
14	280 ♂	0	3510	—	—	—
		7	3300	3910	610	
		15	3000	3950	950	
		30	3070	4050	980	
15	220 ♀	0	2420	—	—	New glucose-1-phosphate used
		15	770	1870	1100	
		30	520	1880	1360	
	Means	0	4590	—	—	
		15	3990	4800	810 (±90)*	
		30	3800	4720	920 (±180)*	

* Standard error in brackets.

Table 2. *Glycogen production by minced normal muscle*

			Glycogen content (mg./100 g.)		
Exp. no.	Wt. of rat (g.)	Time (min.)	Without additions	With addition of glucose-1-phosphate	Glycogen produced (mg./100 g.)
4	230 ♂	0	690	—	—
		15	480	1060	580
		30	430	780	350
5	215 ♀	0	790	—	—
		15	600	610	10
		30	520	620	100
12	180 ♀	0	510	—	—
		30	350	590	240
13	250 ♂	0	260	—	—
		7	240	390	150
		15	160	280	120
		30	60	140	80
14	280 ♂	0	440	—	—
		7	340	530	190
		15	200	470	270
		30	230	430	200
	Means	0	540	—	—
		15	360	600	240 (±130)*
		30	400	510	110 (±80)*

* Standard error in brackets.

muscle shows only a little glycogenolysis in the first 30 min. after death, but in minced muscle, diluted with 0·8 % NaCl, the glycogen content decreases rapidly. Identical glycogen values were obtained with whole muscle, hydrolyzed directly, or with muscle which was first minced and then immediately hydrolyzed by hot NaOH. The glycogen content of different muscles varies widely (Noll & Becker, 1936). We therefore used the muscles of the abdominal wall, because they present a relatively large quantity of muscle of uniform glycogen content. It was found that the error of glycogen estimation for amounts under 100 mg./100 g. was about 20 %, between 100–500 mg./100 g. about 10–20 %, and between 500–5000 mg./100 g. it was under 10 %.

In all experiments, unless otherwise stated, the amounts of glucose-1-phosphate and of glycogen added were equivalent to a concentration of 3 g. and 1 g./100 g. minced tissue respectively.

1. *Normal fed animals*

Table 1 shows that in all 5 animals minced liver produced glycogen from glucose-1-phosphate. In Exps. 4, 5, 14 and 15 the maximum was reached after 30 min. and in Exp. 13 after 15 min. The greatest glycogen production was seen in Exp. 15 (1360 mg./100 g.). In 0·8 % NaCl the glycogen content decreased during the same period from 2420 to 520 mg./100 g.

Whilst, as shown in Table 1, the glycogen content of liver was between 2·4 and 6·5 %, the glycogen content of abdominal wall muscle was only 0·26–0·79 % (Table 2).

In the five experiments of Table 2, some of which were done on the same animals as in Table 1, addition of glucose-1-phosphate caused the greatest glycogen production in Exp. 4 (580 mg./100 g.) and this was reached after 15 min. Without glucose-1-phosphate the glycogen content decreased from 690 to 480 mg./100 g.

2. *Normal starved animals*

The following experiments were made in order to study the glycogen production by muscle and liver tissue from rats whose glycogen content had been decreased by fasting. Such experiments were required, since in adrenalectomized animals the glycogen content of both liver and muscle is very much decreased, and it appeared possible that this factor might influence glycogen production.

In rats, fasting for 24 hr. reduces the glycogen content of the liver to very low values (FitzGerald, 1938; FitzGerald, Laszt & Verzár, 1938; Verzár, 1939; Kreienberg & Wiesenhüter, 1943), while in muscles the decrease is less (Kreienberg & Wiesenhüter, 1943).

Table 3. *Glycogen production by minced liver from starved animals*

			Glycogen content (mg./100 g.)				Glycogen produced from glucose-1-phosphate		
Exp. no.	Wt. of rat (g.)	Time (min.)	Without additions	With addition of glucose-1-phosphate	With addition of glycogen	With addition of glycogen and glucose-1-phosphate	Without addition of glycogen	With addition of glycogen	Period of starvation (hr.)
6	230 ♂	0	100	—	—	1310	—	—	24
		15	60	80	960	1350	20	390	
		30	80	—	—	1360	—	—	
7	260 ♂	0	70	—	1070	—	—	—	24
		15	20	5	900	970	−15	70	
		30	10	5	880	880	− 5	0	
8	200 ♂	0	—	—	1420	—	—	—	24
		15	—	—	990	1700	—	710	
		30	60	210	810	1350	150	540	
10	200 ♂	0	730	—	—	1600	—	—	24
		15	560	890	1280	1850	330	570	
		30	380	—	1200	1260	—	60	
22	180 ♀	0	40	—	1040	—	—	—	48
		15	20	30	800	1130	10	330	
23	170 ♀	0	30	—	1050	—	—	—	48
		15	20	30	800	1000	10	200	
24	245 ♂	0	160	—	1140	—	—	—	48
		15	90	700	910	1520	610	610	
26	240 ♂̸	0	180	—	1160	—	—	—	72
		15	50	670	680	1360	620	680	
27	265 ♂̸	0	30	—	1030	—	—	—	48
		15	20	120	1070	1390	100	320	
25	260 ♂̸	0	50	—	1070	—	—	—	48
		15	20	610	770	1240	590	470	
28	325 ♂̸	0	80	—	1130	—	—	—	72
		15	30	310	880	1430	280	550	
	Means	0	150	—	1120	—	—	—	
		15	110	340	920	1360	230 (±80)*	440 (±60)*	

* Standard error in brackets.

Table 4. *Glycogen production by minced muscle from starved animals*

			Glycogen content (mg./100 g.)				Glycogen produced from glucose-1-phosphate		
Exp. no.	Wt. of rat (g.)	Time (min.)	Without additions	With addition of glucose-1-phosphate	With addition of glycogen	With addition of glycogen and glucose-1-phosphate	Without addition of glycogen	With addition of glycogen	Period of starvation (hr.)
8	200 ♂	0	130	—	1140	1110	—	−30	24
		15	—	—	1080	1430	—	350	
		30	60	50	910	1520	−10	610	
9	210 ♂	0	170	110	—	—	−60	—	24
		15	160	180	1280	1460	20	180	
		30	100	—	1200	1450	—	250	
10	200 ♂	0	120	50	1000	1050	−70	50	24
		15	40	90	760	1610	50	850	
		30	50	—	490	1250	—	760	
22	180 ♀	0	40	—	1070	—	—	—	48
		20	10	20	850	1280	10	430	
23	170 ♀	0	30	—	1020	—	—	—	48
		15	20	30	870	1410	10	540	
24	245 ♂	0	60	—	1120	—	—	—	48
		20	80	110	970	1230	30	260	
25	260 ⚦	0	40	—	1020	—	—	—	48
		20	10	10	760	1230	0	470	
26	240 ⚦	0	40	—	1040	—	—	—	72
		20	30	130	910	1350	100	440	
27	265 ⚦	0	30	—	1040	—	—	—	48
		20	30	60	780	1100	30	320	
28	325 ⚦	0	30	—	1020	—	—	—	72
		20	20	30	870	1060	10	190	
	Means	0	70	—	1050	—	—	—	
		15	40	70	910	1320	30 (±10)*	410 (±20)*	

* Standard error in brackets.

From Tables 3 and 4 it can be seen that if the glycogen content of liver or muscle is less than 100 mg./100 g., no or almost no production of glycogen from glucose-1-phosphate is observed *in vitro*. Only in Exps. 25 and 28 (Table 3) is glycogen produced in normal quantities by liver, in spite of a low initial glycogen content. Cori & Cori (1940) have shown that the addition of some glycogen to tissue extracts of very low initial glycogen content initiates glycogen formation from glucose-1-phosphate. We therefore added 1 g. glycogen/100 g. liver or muscle in addition to glucose-1-phosphate. This addition of glycogen caused normal quantities of glycogen to be formed, thus confirming Cori's observation.

3. *Adrenalectomized animals*

The animals were killed in severe adynamia. In Tables 5 and 6 + + + + means a state in which the animals would have died in a few hours, while + + + indicates that the rats were still moving but unable to climb, and were falling from a vertical wire netting in 0·5–2 min.

Table 5 shows that the glycogen content of the liver was extremely low and in Exps. 2, 3, 8, 9, 10 and 11 no glycogen was produced by addition of glucose-1-phosphate alone. However, addition of 1 g. glycogen/100 g. liver produced in the same experiments just as much new glycogen as with tissue from normal animals. This glycogen production varied between 150 and 570 mg./100 g. in 15 min. Only in rat no. 7 was the glycogen content of the liver as high as in normal animals, in spite of a distinct adynamia. Without addition of glycogen 470 mg./100 g. of glycogen were produced from glucose-1-phosphate, whilst with addition of glycogen 890 mg./100 g. new glycogen were produced from the phosphate ester. Rat no. 6, in spite of an extremely low glycogen content, produced without addition of glycogen 200 mg./100 g., and with addition of glycogen 400 mg./100 g., of new glycogen from glucose-1-phosphate.

Thus the production of glycogen by tissue from these adrenalectomized animals was not diminished as compared with that with tissue from normal, starved rats. These ten experiments do not indicate any marked effect of adrenalectomy on glycogen

Table 5. *Glycogen production by minced liver from adrenalectomized rats*

Exp. no.	Wt. of rat (g.)	Time (min.)	Glycogen content (mg./100 g.)				Glycogen produced from glucose-1-phosphate		Days after adrenalectomy, and degree of adynamia
			Without additions	With addition of glucose-1-phosphate	With addition of glycogen	With addition of glycogen and glucose-1-phosphate	Without addition of glycogen	With addition of glycogen	
2	195 ♂	0	50	—	—	—	—	—	17
		7	20	10	—	—	−10	—	+ + + +
		15	10	10	—	—	0	—	
		30	20	20	—	—	0	—	
3	190 ♂	0	10	—	—	—	—	—	18
		15	10	5	—	—	−5	—	+ + +
		30	5	5	—	—	0	—	
4	225 ♂	0	—	—	1220	—	—	—	19
		7	—	—	900	1470	—	570	+ + +
		15	—	—	920	1490	—	570	
5	200 ♂	0	20	—	—	—	—	—	18
		7	—	—	820	1300	—	480	+ + +
		30	—	—	680	1300	—	620	
8	130 ♂	0	30	—	1030	—	—	—	10
		15	10	40	—	1320	30	(320)	+ + +
9	150 ♂	0	20	—	1030	—	—	—	10
		15	30	40	930	1080	10	150	+ + +
10	150 ♂	0	20	—	1020	—	—	—	11
		15	20	10	970	1150	−10	180	+ + + +
11	145 ♂	0	40	—	—	—	—	—	11
		15	30	60	1010	1270	30	260	+ + +
6	117 ♂	0	20	—	1030	—	—	—	5
		15	20	220	830	1230	200	400	+ + +
7	102 ♂	0	2000	—	2900	—	—	—	5
		15	1850	2320	2220	3110	470	890	+ + +
	†Means	0	30	—	1070	—	—	—	
		15	20	60	930	1260	40 (±30)*	330 (±80)*	

* Standard error in brackets.
† Nos. 5 and 7 are not included.

formation from glucose-1-phosphate by liver tissue. Many more experiments would be necessary to reveal possible differences of velocity or slight, statistically significant, differences.

Table 6 shows that muscles of the same adrenalectomized animals too have very low initial glycogen contents. In none of these experiments was there a production of glycogen from glucose-1-phosphate, without addition of extra glycogen. If, however, 1 g. glycogen/100 g. muscle was added, new glycogen was produced in about the same quantity as with starved normal animals. Thus the muscle from adrenalectomized animals too, has not lost the capacity to produce glycogen from glucose-1-phosphate.

DISCUSSION

The above experiments show that normal minced liver and muscle produce glycogen from glucose-1-phosphate. In starved rats with very low glycogen content, however, no glycogen is produced by minced muscle or liver, unless glycogen is added to the solution. This seems to show that the glycogen-producing enzyme (phosphorylase, Cori *et al.* 1942) can only produce glycogen from glucose-1-phosphate if some glycogen is already present. Kiessling (1939*a*, 1939*b*), Cori, Cori & Schmidt (1939) and Colowick & Sutherland (1942) have obtained similar results with pure enzyme solutions. Phosphorylase thus appears to increase the size of glycogen molecules which are already present without increasing their number.

It is well known that adrenalectomized rats show a very low glycogen content in the liver and later also in the muscles. Since starvation for 24–48 hr. in rats produces similarly low glycogen values, it seemed possible that the low glycogen contents of adrenalectomized rats are caused, at least partly, by the concomitant starvation. Liver or muscle tissue from adrenalectomized animals produces no glycogen without addition of extra glycogen and thus behaves like that from starved animals. But glycogen is produced in the same quantities as by tissue from normal animals, if glycogen is added. Thus the disturbance of carbohydrate metabolism

Table 6. *Glycogen production by minced muscle from adrenalectomized animals*

			Glycogen content (mg./100 g.)				Glycogen produced from glucose-1-phosphate		
Exp. no.	Wt. of rat (g.)	Time (min.)	Without additions	With addition of glucose-1-phosphate	With addition of glycogen	With addition of glycogen and glucose-1-phosphate	Without addition of glycogen	With addition of glycogen	Days after adrenalectomy and adynamia
2	195 ♂	0	30	—	—	—	—	—	17
		7	20	—	—	—	—	—	+ + + +
		15	10	10	—	—	0	—	
		30	10	10	—	—	0	—	
3	190 ♂	0	20	—	—	—	—	—	18
		7	10	10	—	—	0	—	+ + +
		15	10	5	—	—	- 5	—	
		30	5	0	—	—	- 5	—	
5	200 ♂	0	40	—	—	—	—	—	18
		7	—	—	1090	1340	—	250	+ + +
		30	—	—	900	1300	—	400	
4	225 ♂	0	—	—	1170	—	—	—	19
		7	—	—	970	1240	—	270	+ + +
		30	—	—	930	1150	—	220	
6	117 ♂	0	30	—	1070	—	—	—	5
		20	—	40	940	1700	(10)	760	+ + +
7	102 ♂	0	80	—	—	—	—	—	5
		20	—	100	—	2240	(50)	(1000)	+ + +
8	130 ♂	0	25	—	—	—	—	—	10
		20	15	25	1000	1420	10	420	+ + +
9	150 ♂	0	110	—	1150	—	—	—	10
		20	20	10	1010	1320	-10	310	+ + +
10	150 ♂	0	30	—	—	—	—	—	11
		20	20	20	930	1350	0	420	+ + + +
11	145 ♂	0	60	—	—	—	—	—	11
		20	30	70	950	1250	40	300	+ + +
	†Means	0	50	—	—	—	—	—	
		20	20	40	950	1360	20 (±10)*	410 (60)*	

* Standard error in brackets.
† No. 7 is not included.

after adrenalectomy cannot be due to a lack of glycogen production from glucose-1-phosphate. This result is in agreement with the observation with surviving diaphragms of rats (Koepf *et al.* 1941; Verzár & Wenner, 1948) that there is no difference as regards glycogen formation from glucose between diaphragms obtained from normal and adrenalectomized animals. Thus only minced muscle seems to be unable to produce glycogen when the initial glycogen content is low, since undamaged whole muscle retains the ability to produce glycogen under these conditions. The surviving diaphragm produces glycogen from glucose, but not from glucose-1-phosphate (Verzár & Wenner, 1948). This is not incompatible with the present finding that minced liver and muscle produce glycogen from glucose-1-phosphate. The latter, unlike glucose, does not diffuse through cell membranes into the cells of the undamaged surviving diaphragm. Glucose-1-phosphate, like other phosphoric acid esters of glucose, is produced inside the cells. The production of those esters inside the cell is, as pointed out by Verzár & McDougall (1936) and Minibeck & Verzár (1940), the factor responsible for the increased diffusion rate of glucose through cell membranes. In minced liver and muscle most of the cells are damaged. The greater part of the glycogen is not produced inside the cells but by dissolved enzymes in the solution. This may explain the fact that addition of 1% glycogen to the solution increases glycogen production from glucose-1-phosphate. Moreover, it is known that with pure solutions of phosphorylase production of new glycogen takes place only when some glycogen is already present.

The result that muscle and liver tissue from adrenalectomized animals has not lost the ability to form new glycogen is in conformity with the finding that the surviving diaphragm of adrenalectomized rats produces glycogen (Koepf *et al.* 1941; Verzár & Wenner, 1948). The disturbance of carbohydrate metabolism after adrenalectomy is thus due either to an inhibition of phosphorylation, or, more probably, to a disturbance of the breakdown of glycogen and not of its synthesis.

SUMMARY

1. Minced muscle and liver of normal rats produce glycogen from glucose-1-phosphate *in vitro*.

2. If, after a starvation period, the glycogen content falls below 200 mg./100 g., minced liver and muscle do not produce glycogen from glucose-1-phosphate. However, if glycogen is added as well, additional glycogen is produced, just as with pure enzyme solutions.

3. In adrenalectomized, adynamic rats the glycogen content is decreased in liver and muscle, and only if glycogen is added can production of new glycogen by minced muscle and liver be observed to occur at the same rate as with normal animals. Thus adrenalectomy does not affect the mechanism responsible for the synthesis of glycogen from glucose-1-phosphate.

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The Action of Steroids on Glycogen Breakdown in Surviving Muscle

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In a previous paper (Verzár & Wenner, 1948) the effect of deoxycorticosterone (DOC) on glycogen synthesis from glucose with or without insulin was studied in the surviving diaphragm of the rat. DOC in concentrations of 5 mg./100 ml. of suspending fluid counteracts the effect of insulin and increases glycogen breakdown. In order to establish whether this effect of DOC is specific, several other steroids, sex hormones and similar biologically active and inactive substances have been tested in the same way.

METHODS

The technique used was that of Gemmill (1940, 1941) and of Gemmill & Hamman (1941) with modifications as described in our previous paper (Verzár & Wenner, 1948). The diaphragms of rats were incubated in oxygenated, phosphate buffer Ringer solution containing 100 mg. glucose and 1 unit insulin/100 ml. of solution. The steroid was added in a 2% (w/v) solution in ethanol or in a 1% solution in 50% (v/v) ethanol, if this was possible without crystallization occurring. Thus 0·05 or 0·1 ml. had to be added to 10 ml. solution to give a concentration of 5 or 10 mg. steroid/100 ml. solution.

Surface tension was measured with the Traube stalagmometer (Traube, 1887; Abderhalden, 1930). For these experiments the 50% ethanolic solutions of the steroids were diluted with distilled water to a concentration of 5 or 10 mg./100 ml. The surface tension is expressed in dynes/cm. (Landolt-Börnstein, 1923).

RESULTS

Table 1 shows the effect of various steroids on glycogen breakdown. Each line contains the mean values of a series of at least 4, but mostly of 8 or more single experiments. Steroids 1–14 contain either free keto or hydroxyl groups or both, whilst steroids 15–17 contain either an ether or an ester linkage. Substances 18 and 19 are not related to steroids. The effect on glycogen breakdown is seen from a comparison of columns 2 and 3. The dose of 5 mg./100 ml. was chosen to show the different effects of the steroids. DOC in a concentration of 5 mg./100 ml. of solution inhibits glycogen production in the presence of 100 mg. sugar and 1 unit insulin/100 ml. solution. Progesterone (no. 2) and pregnenediolone (no. 4) show even a larger effect. With 5 mg. progesterone the glycogen content is decreased even below the initial value.

Pregnanediolone, corticosterone, hydroxycorticosterone, testosterone, androstanediol (nos. 5, 6, 7, 8, 9) have a distinctly smaller effect. 11-Dehydrocorticosterone (no. 17) was also active; it could only be tested as acetate. Pregnanediolone, testosterone and androstanediol were, in the higher concentration (10 mg./100 ml.), as active as DOC; but oestradiol (no. 10) and bisdehydrodoisynolic acid (no. 11) had the same activity at a concentration of 10 mg./100 ml. as the former group (nos. 5–9) had at a concentration of 5 mg./100 ml. Hydroxyaetiocholenic acid (no. 12), *allo*pregnanepentol (no. 13), and androstenediol (no. 14) had no action even in the higher concentration of 10 mg./100 ml. Stilboestrol (no. 18), an oestrogen, was more active than oestradiol and its activity was equal to that of progesterone or DOC.

The glucoside of DOC (no. 15) had no activity, while the monoacetate of pregnenediolone (no. 16) had about the same activity as the non-esterified substance (no. 4). The acetate of 11-dehydrocorticosterone was also very active. Oleic acid (no. 19), with a molecular weight similar to those of the steroids tested, was without activity.

DISCUSSION

The inhibition of glycogen production and increase of glycogen breakdown is not present in substances lacking a hormone-like action such as hydroxyaetiocholenic acid and oleic acid. On the other hand high activity was found in stilboestrol and, to a lesser extent, in bisdehydrodoisynolic acid, substances which are chemically different and only related biologically to the steroid hormones.

In the DOC-21-glucoside (no. 15) the new linkage on the 21-hydroxyl group abolished the whole activity. It is possible that this substance cannot be split by intact muscle, but the acetate of pregnenediolone (no. 16) was as active as pregnenediolone itself. The acetate of 11-dehydrocorticosterone (no. 17) was also active. Thus, if the above explanation is correct, muscle must be able to split these two esters. It should be remembered that in previous experiments on glycogen phosphorylation (Montigel & Verzár, 1943) it was found that steroids esterified in the 21-position were less active.

It is much more difficult to explain the differences between the activities of the various other substances on glycogen breakdown. It might appear that the 11-hydroxysteroids which are more soluble in water have a smaller activity. Thus the most active substances progesterone (no. 2), pregnenediolone (no. 4) and stilboestrol (no. 18), which are active even in a concentration of 1 mg./100 ml., have a smaller solubility in water than corticosterone (no. 6) and *allo*pregnanepentol (no. 13), which are less active. On the other hand oestradiol (no. 10) and androstenediol (no. 14) are less soluble in water than progesterone (no. 2), but have a smaller activity than corticosterone (no. 6). Thus differences in water solubility do not explain the greater activity of certain steroids on the inhibition of glycogen formation.

Another explanation might be found in the surface activity of steroids (Sobotka, 1938). The steroids, after adsorption on the cell surface might inhibit the diffusion of glucose or of oxygen into the cells. The substances most active in glycogen breakdown, such as DOC and pregnenediolone, lower the surface tension of water to 68 and 68·5 (dynes/cm.) respectively; the inactive oestradiol had in aqueous solution a surface tension of 70; and the fairly active corticosterone 58·5. Thus there was no correlation between inhibition of glycogen formation and surface tension.

There is a marked difference in inhibitory action on glycogen production between DOC and progesterone, both of which are very active, and corticosterone and 17-hydroxycorticosterone which show very little activity. The second group also has a distinct action on glycogen production in the whole animal and this is actually used in some assays of hormones of the adrenal cortex, such as the work test of Ingle (1941) and the glycogen deposition test (Reinecke & Kendall, 1942; Dorfman, Ross & Shipley, 1946; Eggleston, Johnston & Dobriner,

1948

1946). Many authors therefore believe that 11-hydroxysteroids have a specific effect on carbohydrate metabolism, whilst DOC has none. This point has been discussed by us (Verzár, Bucher, Somogyi & Wirz, 1941), and we consider that there is a difference in the velocity but not in the nature of the action of the two groups of steroids. It is remarkable that the results described in this paper show differences between these two groups also with respect to glycogen metabolism *in vitro*.

It has been known for a long time that the sex hormones show effects on carbohydrate metabolism which are comparable with those of the adrenal cortical hormones. Montigel & Verzár (1943) have shown that progesterone, testosterone and to a smaller degree oestradiol influence phosphorylation of glycogen by muscle. The activity is of the same order as that observed in the present experiments. Ingle (1940, 1943, 1944) and Janes & Nelson (1940) have demonstrated that the naturally occurring sex

Table 1. *The effects of various steroids and related substances on the glycogen content of rat's diaphragm in the presence of glucose and insulin*

	Steroid			Glycogen content (mg./100 g.)				
					After 1·5 hr. with addition of glucose and insulin			
No.	Name	Concentration (mg./100 ml.)	No. of animals	Initial 1	Without steroid 2	With addition of steroid 3	Mean difference (mg./100 g.) 3–2	S.E. ± (mg./100 g.)
1	Deoxycorticosterone	10	21	213	309	156	−153	18
		5	30	246	380	244	−136	13
		1	20	287	422	402	− 20	24
2	Progesterone	10	12	325	410	237	−173	27
		5	8	359	456	252	−204	30
		1	7	283	400	308	− 92	36
3	17-Hydroxyprogesterone	10	7	209	443	241	−202	53
4	Pregn-5-ene-3(β):21-diol-20-one	10	4	275	405	212	−193	20
		5	4	290	475	305	−170	20
		1	12	287	415	368	− 47	14
5	Pregnane-3(α):12(β)-diol-20-one	10	8	305	412	285	−127	22
		5	8	356	455	392	− 63	21
6	Corticosterone	5	16	339	433	367	− 66	16
7	17-Hydroxycorticosterone	5	8	331	485	441	− 44	27
		1	8	260	365	355	− 10	37
8	Testosterone	10	8	405	536	292	−244	20
		5	8	281	384	312	− 72	25
		1	8	200	360	228	−132	36
9	Androstane-3(β):17-diol	10	6	423	527	395	−132	52
		5	12	257	389	320	− 69	18
10	Oestradiol	10	8	201	360	278	− 82	15
		5	8	190	344	334	− 10	24
11	Bisdehydrodoisynolic acid	10	8	254	420	310	−110	18
		5	8	192	351	355	+ 4	22
12	3(β)-Hydroxyaetiochol-5-enic acid	10	8	309	366	381	+ 15	18
13	*allo*Pregnane-3(β):11(β):17:20:21-pentol	10	7	285	360	321	− 39	33
14	Androst-5-ene-3(β):17-diol	10	8	280	428	400	− 28	35
		5	8	269	421	385	− 36	13
15	Deoxycorticosterone-β-*d*-glucoside	10*	20	252	364	336	− 28	26
16	Pregn-5-ene-3(β):21-diol-20-one-21-acetate	10	7	237	351	214	−137	28
17	11-Dehydrocorticosterone-acetate	10	8	340	462	370	− 92	24
18	Stilboestrol (4:4′-dihydroxy-αβ-diethylstilbene)	10	6	202	302	148	−154	37
		5	8	199	348	204	−144	27
		1	10	206	310	252	− 58	28
19	Oleic acid (Na salt)	10	4	438	655	790	+ 45	33
		5	4	358	562	594	+ 35	34

* Calculated as pure DOC.

hormones and stilboestrol have a marked influence on the sugar excretion of pancreatectomized rats. It has also been shown (Kochakian, 1945–6) that testosterone lowers the activity of the alkaline and enhances the activity of the acid phosphatase of the kidney. Androstanediol has an effect on phosphatases similar to that of testosterone, but androstenediol has no effect.

In our present experiments on glycogen breakdown we found that testosterone (no. 8) was very active, androstanediol (no. 9) less active, while androstenediol (no. 14) had no activity. It is probable that the effect on glycogen breakdown in our experiments, which is especially marked with progesterone and weak with oestradiol, has its parallel in the action of these substances on phosphorylation of glycogen. Thus the experimental results of the inhibition of insulin activity and stimulation of glycogen breakdown in isolated muscle, shown here with DOC and some sex hormones, has its parallel in many other facts. This is not an argument against the specificity of the action of the adrenal cortical hormones on carbohydrate metabolism. Sex hormones appear to act specifically on certain tissues of secondary sexual character, and their main metabolic function might be of the same nature, as was pointed out long ago by one of us. It might be mentioned that we observed that the insulin effect on glycogen synthesis changes throughout the year, which may be connected with varying sex-hormone production in the animals.

It has not been possible to explain how the steroid hormones act on cell metabolism (Long, 1942; Ingle, 1945). It has been suggested that they are 'velocity factors' acting on enzymic reactions of carbohydrate metabolism (Montigel & Verzár, 1943). Another possibility is that the steroids are adsorbed on the surface of the cells and inhibit diffusion of oxygen so that an anaerobic breakdown of glycogen would result. Alternatively, it is possible that the diffusion of glucose from the solution into muscle fibres may be inhibited by the adsorption of the steroids on the cell surface. Whilst such possibilities cannot be denied, an action on intracellular enzymes active in the intermediary carbohydrate metabolism still seems the most probable, although unproven, possibility.

SUMMARY

1. The effect of deoxycorticosterone (DOC) on glycogen breakdown in the intact diaphragm of the rat *in vitro* has been compared with the effect of several other steroid hormones and related substances.

2. Progesterone, 17-hydroxyprogesterone and pregnenediolone had an equal or even stronger effect. Corticosterone, 17-hydroxycorticosterone, testosterone, androstanediol and pregnanediolone showed a smaller effect. Oestradiol, bisdehydrodoisynolic acid, 3-hydroxyaetiocholenic acid, *allo*-pregnanepentol and androstenediol had the smallest activity, whilst stilboestrol showed a very high activity.

3. The steroids with corticoid activity possessing a hydroxyl group in the 11-position were less active than DOC. The biological activities of the steroids tested did not go parallel with their water solubilities, nor did differences in surface activity explain the differing activities of these substances on glycogen breakdown and the inhibition of the effect of insulin on glycogen production. There is, however, a certain parallelism between the activities of these substances towards glycogen breakdown as shown in the present experiments and their effects on glycogen phosphorylation by minced muscle.

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A New Method for the Determination of 1:2-Dithiols

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The use of 1:2-dimercaptopropanol (BAL) for the treatment of systemic arsenic intoxication after massive contamination with arsenical vesicants, and after overdosage with arsenicals during arsenotherapy, makes essential a method of high sensitivity for its determination, especially in biological materials. The only method at present available (Spray, 1944) depends on the colour produced by the addition of cobalt salts to solutions of BAL. This method is rather insensitive and the metallic complex, being insoluble, has to be maintained in solution by the use of a protective colloid, a procedure which gives uncertain results. It is also by no means specific, for cobalt salts give colours with almost all thio compounds (Spray, Stocken & Thompson, 1945). The procedure adopted by Spray (1944) for determination in blood involved ultrafiltration after acidification to stabilize the BAL; by this means 80–85% of BAL was recovered from plasma and some 40–60% from blood. In our opinion any method which depends on the isolation of BAL as such from blood is likely to have little success, it being too unstable in this medium.

EXPERIMENTAL

Principles of method

It has been found that 1:2-dithiols react with cyanogen chloride to produce an unidentified compound which readily liberates thiocyanate in alkaline solution. The determination of thiocyanate in micro-quantities has been previously reported (Aldridge, 1944, 1945).

For its determination in biological materials, we have not attempted the isolation of BAL but have converted it to the much more stable compound of BAL with cyanogen chloride and then recovered this compound by a protein precipitation technique (Folin, 1930).

Reagents

Cyanogen chloride solution. (*a*) For determination of BAL in aqueous solution carefully pipette 0·1 ml. (approx.) cyanogen chloride, using an ice-cold pipette, into 200 ml. of Na acetate (*c.* 1 N). (*b*) For determination of BAL in blood and urine, 0·1 ml. cyanogen chloride in 100 ml. 3·8% (w/v) $Na_2SO_4.10H_2O$. Great care should be exercised in the handling of cyanogen chloride. It is highly toxic and extremely volatile (b.p. 14°) and should be stored in a refrigerator in a bottle with a well greased stopper having an external joint.

Bromine water. (*a*) For determination of BAL in aqueous solution, a half-saturated solution. (*b*) For determination of BAL in blood and urine, saturated solution.

Arsenious acid. 2% (w/v) solution. To obtain complete solution boil under reflux.

N-*Sodium acetate.* 13·6% (w/v) solution of sodium acetate,

$$CH_3.COONa.2H_2O.$$

Sodium tungstate. 10% (w/v) solution $Na_2WO_4.2H_2O$ neutralized to phenolphthalein.

Pyridine reagent. To 1 l. of the constant boiling mixture of pyridine and water (59% pyridine), add 100 ml. of conc. HCl.

Benzidine reagent. 5% (w/v) solution of recrystallized benzidine hydrochloride in 2% (v/v) HCl.

Acids, etc. N-NaOH, 3N-HCl, 0·66N-H_2SO_4.

Reaction of dithiols with cyanogen chloride

The reaction of cyanogen chloride with 1:2-dithiols in aqueous solution has been studied at pH values of 6–10. To 6 ml. 0·05M-phosphate buffer at the required pH (checked by glass electrode) were added 2 ml. cyanogen chloride solution (1 mg.) and 2 ml. BAL solution (70 μg.). At various times, 1 ml. of the above solution was pipetted into 1·8 ml. 0·2N-NaOH. After leaving for 10 min., the solution was rendered acid by the addition of 0·2 ml. 3N-HCl. The thiocyanate formed was estimated in the usual way (Aldridge, 1944, 1945). It is seen from Table 1 that the reaction is complete in 2 min. at pH 7–8 while at pH 6 it requires 15 min. As a convenient acidity for the reaction, pH 7 has been chosen, though any high pH may be used provided sufficient excess of cyanogen chloride is added in order that the reaction between BAL and cyanogen chloride is complete before all the cyanogen chloride is hydrolyzed. The poor stability of BAL at high pH prevents the use of very alkaline solutions.

Table 1. *Speed of reaction of cyanogen chloride with 1:2-dithiols at various acidities*

(Thiocyanate determined at various times.)

Time (min.)	Spekker readings		
	pH 6·0	pH 7·0	pH 8·0
0·5	0·36	0·50	0·495
1	0·41	0·51	0·505
2	0·44	0·54	0·54
4	0·465	0·54	0·54
8	0·52	0·54	0·54
16	0·535	—	—

Cyanogen chloride may be determined in aqueous solution by omitting the bromination stage in the normal thiocyanate method. By a determination of the residual cyanogen chloride after reaction with BAL at various pH, it has been possible to determine the molecular proportions of cyanogen chloride and BAL taking part in the reaction. Within the limits of experimental error, 1 mol. of cyanogen chloride reacts with 1 mol. of dithiol (Table 2).

Table 2. *Determination of molecular proportions of cyanogen chloride and BAL in reaction*

(34 μg. BAL taken and excess cyanogen chloride determined after completion of reaction at various pH. Blank contains all reagents except dithiol.)

	Spekker reading		Cyanogen chloride reacting	Mol. cyanogen chloride
pH	Blank	Dithiol	(μg.)	/mol. BAL
6·32	1·04	0·26	15·9	0·93
6·94	1·04	0·25	16·1	0·94
7·96	1·03	0·20	16·9	0·99
9·95	0·92	0·15	15·7	0·92

As will be shown later, 1:3-dimercaptopropanol does not interfere in the estimation but acts as a reducing agent and cyanogen chloride is converted to cyanide and chloride. The cyanogen chloride which reacts with 1:3-dithiol is determined as given previously, and cyanogen chloride together with HCN is estimated by including the bromination process as for thiocyanate estimation. The results (Table 3) show that all the reacting cyanogen chloride is reduced to cyanide. Examination of BAL in an identical manner indicates that of the 1 mol. of cyanogen chloride reacting with 1 mol. BAL, 0·05 mol. cyanide is produced (Table 3).

Estimation of SH groups present during the reaction by titration in acid solution with iodine shows that after the initial reaction with cyanogen chloride both SH groups are substituted, and that after alkaline treatment to liberate thiocyanate from the compound of BAL with cyanogen chloride, some indication of the reappearance of SH groups is shown (Table 4). The fact that SH groups are present after treatment with NaOH, in which medium dithiols are rapidly oxidized to the disulphide form, is a strong indication that a greater proportion was produced than that titrated.

Table 3. *Reaction of* 1:3-*dimercaptopropanol and BAL with cyanogen chloride at* pH 7

(Cyanogen chloride and cyanogen chloride + HCN determined after completion of reaction. Blank contains all reagents except dithiol.)

	Spekker readings		
Estimation	Blank	Dithiol	
	1:3-Dimercaptopropanol		
CNCl	0·625	0·030	CNCl reacting, 0·98 mol./mol. dithiol
CNCl + HCN	0·630	0·630	—
HCN (by diff.)	0·005	0·600	HCN produced, 0·98 mol./mol. dithiol
	1:2-Dimercaptopropanol (BAL)		
CNCl	0·980	0·290	CNCl reacting, 1·01 mol./mol. dithiol
CNCl + HCN	1·010	0·355	—
HCN (by diff.)	0·030	0·065	HCN produced, 0·052 mol./mol. dithiol

Table 4. *Determination of SH groups during BAL-cyanogen chloride reaction*

(SH groups titrated with 0·01 N-I_2 in acid solution.)

Time of estimation	Titration (ml. 0·01 N-I_2)	Equivalent SH groups
Initial	1·58	1·96
After reaction with CNCl	0·02	—
After alkaline treatment	0·16	

Alkaline treatment of the compound of BAL with cyanogen chloride

The reaction product between cyanogen chloride and 1:2-dithiols, although stable in acid solution, liberates thiocyanate completely 5 min. after making alkaline with NaOH. Thiocyanate is also liberated, though more slowly, at pH values 9–10 (Table 5).

The excess cyanogen chloride left in solution is also destroyed by treatment with alkali, being in the main converted to sodium cyanate. However, a small percentage is converted to cyanide (Aldridge, 1945) which would of course react in the same manner as thiocyanate in the subsequent determination. The cyanide is readily removed by making the solution acid after alkaline treatment for 10 min. and bubbling air through the solution for 30 min. (air saturated with water vapour is used to prevent evaporation of the solution). This has no effect on the thiocyanate (Aldridge, 1944). With a blank determination, adding all the reagents except the dithiol, a zero reading on the Spekker absorptiometer is obtained with Ilford spectrum green 604 filters.

It has been shown that a small proportion of cyanide is liberated together with thiocyanate during the alkaline treatment. To determine the amount of cyanide, solutions of the BAL-cyanogen chloride compound were rendered acid and aerated to remove excess cyanogen chloride. Thiocyanate and cyanide were then estimated by the normal procedure and thiocyanate alone after removal of the cyanide by further aeration. Table 6 shows that while about 0·8 mol. thiocyanate are liberated from 1 mol. BAL, 0·05 mol. cyanide is also produced.

It should be noted that after the reaction of 1:2-dithiols with cyanogen chloride there are two possible procedures: (*a*) render the solution acid and aerate to remove excess cyanogen chloride, before liberating the thiocyanate, or (*b*) remove cyanide produced during destruction of excess

Table 5. *Liberation of thiocyanate from BAL-cyanogen chloride compound at various acidities at room temperature*

Caustic alkali			pH 9			pH 10·0		
Time (min.)	Spekker reading	% of total	Time (min.)	Spekker reading	% of total	Time (min.)	Spekker reading	% of total
1	0·390	93	3	0·037	8·5	3	0·110	25
2	0·400	95	6	0·052	12	6	0·130	29·5
4	0·405	96	12	0·065	15	12	0·185	42
10	0·410	98	24	0·080	18·5	24	0·255	58
16	0·410	98	48	0·112	26	48	0·315	72
24 hr.	0·420	100						

Table 6. *Alkaline treatment of BAL-cyanogen chloride compound*

(Excess cyanogen chloride removed by aeration in acid solution followed by alkaline treatment. Thiocyanate + cyanide and thiocyanate estimated.)

	Spekker readings	
Estimation	Blank	Dithiol
HCNS + HCN	0·052	0·790
HCNS	0·020	0·720
HCN (by diff.)	0·030	0·070

—

HCNS produced, 0·79 mol./mol. BAL
HCN produced, 0·05 mol./mol. BAL

cyanogen chloride with alkali as given in the description of the method. Procedure (*a*) is useful in the elimination of interference of ergothioneine and gives slightly higher results than (*b*) owing to the extra 0·05 mol. cyanide/mol. BAL (Table 6). Procedure (*b*) has been adopted because the removal of the relatively large quantity of cyanogen chloride by (*a*) requires rather long aeration.

Specificity of the reaction

In order to check the specificity of the reaction for 1:2-dithiols, a large number of compounds has been examined for interference and especially those likely to be encountered in biological fluids. The following is a list of those tested:

1:3-Dimercaptopropanol
Hexane-1:6-dithiol
Monothioethylene glycol
BAL in disulphide form
Cysteine hydrochloride
Cystine
Thiodiglycol
Thioglycollic acid
Methionine
Ethyl sulphide
n-Butyl sulphide
Thiophenol
Ethyl mercaptan
Ergothioneine
Glutathione
Thiolacetic acid

Table 7. *Speed of reaction of cyanogen chloride with ergothioneine at various acidities*

(Cyanogen chloride allowed to react with ergothioneine for 20 min. Estimation as for that of BAL.)

pH	Spekker reading
7·2	0·02
8·06	0·02
9·0	0·04
10·0	0·150

The general method we have adopted for the examination of such compounds has been to treat 50 and 30 μg. of the compounds (*c*. 10 μg. of BAL gives a Spekker reading of 1·0) as for the estimation of 1:2-dithiols. In addition, a blank was run with 50 μg. of the compound and all the reagents except cyanogen chloride; this checks for the presence of thiocyanate and any other similar interfering substances. With the above procedure, all except three of the compounds listed in the text gave zero readings: glutathione, ergothioneine and thiolacetic acid. It is not at all clear why glutathione reacts; 40 μg. gives a Spekker reading of 0·09 (*c*. 1 μg. of BAL gives the same reading). It is possible that this result is due to some impurity in the sample of glutathione examined. Ergothioneine reacts slowly with cyanogen chloride at high pH values and to a small extent up to acidities of pH 9 (Table 7). Interference of ergothioneine in the method must therefore take place when NaOH is added to liberate thiocyanate and to destroy excess cyanogen chloride. If necessary, interference from ergothioneine may be prevented by removing excess cyanogen chloride by a stream of air before making alkaline as mentioned previously. However since ergothioneine is not recovered from blood with the protein precipitation procedure to be described, the need for this alteration in the technique is not common. In amounts of 12 μg., thiolacetic acid gives a Spekker reading of 0·45 (Table 8). This is about 25% of the theoretical value expected if 1 mol. of thiocyanate should be obtained from each mol. thiolacetic acid. It is probable that, in the highly alkaline solution, sulphide is produced which reacts with the excess cyanogen chloride or the small amount of cyanide liberated during the hydrolysis of cyanogen chloride.

Table 8. *Interference of thiolacetic acid in the method*

(Various amounts of thiolacetic acid treated as for BAL estimation. Recoveries are based on the theoretical liberation of 1 mol. HCNS/mol. thiolacetic acid.)

Thiolacetic acid (μg.)	Spekker reading	Recovery (%)
6·05	0·220	22·5
12·1	0·455	23·3
18·2	0·605	20·8
24·2	0·835	21·4
30·3	1·03	21·1

Procedure for the estimation of 1:2*-dithiols*

Description. To 2 ml. of a solution containing not more than 10 μg. of a 1:2-dithiol, add 0·4 ml. of the cyanogen chloride reagent, mix and allow to stand for 5 min. Add 0·4 ml. N-NaOH and allow to stand for 10 min. Make acid by the addition of 0·2 ml. 3N-HCl and bubble air saturated with water vapour through the solution for 30 min. Add 0·2 ml. bromine water, mix and remove excess bromine with 0·2 ml. arsenious acid. Mix well, blow out any bromine vapour left in the tube, and pour into a previously prepared mixture of 4 ml. pyridine reagent and 0·8 ml. benzidine reagent (total volume of solution 8·2 ml.). Mix well by pouring back and forth between the tubes. Allow the colour to develop for 20 min. and then read on the Spekker absorptiometer or any other photoelectric instrument with 1 cm. cells and Ilford spectrum green 604 filters. It should be noted that throughout this work syringe pipettes (maximum error 0·2 %) have been used. With the numerous additions of small quantities of reagents great accuracy in measurements is essential. In order to prevent the catalytic oxidation of the dithiols due to the presence of traces of metals in the distilled water, special precautions have been taken in preparing the standard solutions for the determination of the calibration curves. The solvent used was distilled water, saturated with CO_2, and to which 10 μg. Na cyanide/ml. were added to poison the metallic catalysts. This procedure seems effectively to prevent the oxidation. The figures obtained for the calibration curves for three dithiols are given in Table 9.

Table 9. *Calibration curves for* 1:2*-dimercaptopropionic acid,* 1:2*-propane dithiol and BAL*

(Recoveries are based on the theoretical liberation of 1 mol. thiocyanate/mol. of dithiol. Photometric readings (Ilford spectral green 604 filters, 1 cm. cells).)

Amount (μg.)	Spekker reading	Recovery (%)
	1:2-Dimercaptopropionic acid	
2·20	0·180	92
4·41	0·350	90
6·61	0·520	89
8·81	0·690	88
11·02	0·860	88
	1:2-Propane dithiol	
3·33	0·305	81
6·66	0·610	81
9·99	0·905	80
	1:2-Dimercaptopropanol (BAL)	
2·31	0·175	77
4·62	0·550	77
6·93	0·540	79
9·24	0·720	79
11·56	0·905	79

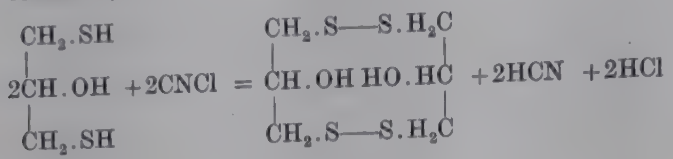
Application to biological fluids. Pipette as soon as possible 1 ml. of whole blood into 1·5 ml. cyanogen chloride reagent in isotonic Na sulphate contained in a centrifuge tube. Leave for 10 min., then add a further 1 ml. cyanogen chloride reagent and leave for 5 min. After this time, add 0·5 ml. 10 % sodium tungstate, mix well and follow by 0·5 ml. 0·66N-H_2SO_4. Mix again and centrifuge for 10 min. The same procedure is satisfactory for BAL in plasma. It is essential to have the blood in contact with the cyanogen chloride as soon as possible to minimize losses of BAL due to decomposition. The procedure used for urine is as given above for blood except that 1 ml. N-sodium acetate is added to the cyanogen chloride reagent before the addition of the urine and no tungstate or sulphuric acid is required. The procedure thenceforward is identical for blood and urine. To not more than 2·0 ml. of filtrate from blood protein precipitation or urine solution add 0·4 ml. N-NaOH and leave for 10 min. Acidify with 0·2 ml. 3N-HCl, make up to 2·6 ml. with water and bubble air saturated with water vapour through the solution for 30 min. to remove cyanide. Brominate with 0·4 ml. bromine water, removing excess with 0·4 ml. arsenious acid. Mix well, blow out any bromine vapour and continue as given under 'description'. For the thiocyanate blank determination, the above procedure is followed replacing the cyanogen chloride reagent with the same volume of isotonic sodium sulphate solution, i.e. 2·5 ml. The difference between the two determinations gives the 1:2-dithiol concentration (plus any interfering substances) and should be referred to the calibration curve as determined before.

DISCUSSION

Mechanism of reaction

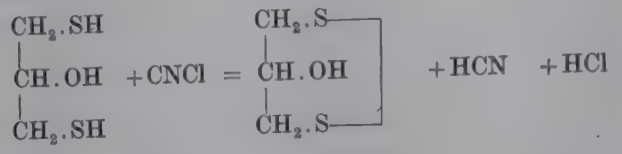
Of the theoretical yield of thiocyanate based on a reaction of 1 mol. of cyanogen chloride with 1 mol. of BAL, 78 % is obtained (Table 9). Iodine titration of the BAL gave 98·6 % purity. This titration includes substances such as 1:3-dimercaptopropanol but excludes substances such as the oxidized form of BAL. It has been shown that 1 mol. cyanogen chloride reacts with 1 mol. BAL (Table 2) and that 0·8 mol. of thiocyanate is easily liberated in alkaline solution (Table 5); slowly at pH 9 and quickly in NaOH. 1:3-Dimercaptopropanol also reacts with cyanogen chloride but no thiocyanate is liberated in alkaline solution; however, cyanide is liberated equivalent to the cyanogen chloride which has reacted (Table 3). Determination of SH groups during the reaction has shown that both are substituted after the reaction with cyanogen chloride, but after the alkaline treatment they reappear to a small extent (0·2 SH groups/molecule of BAL (Table 4)).

Since cyanide is produced equivalent to the amount of cyanogen chloride reacting with 1:3-dimercaptopropanol, it would seem that it is a simple reduction of cyanogen chloride to cyanide and chloride, i.e.



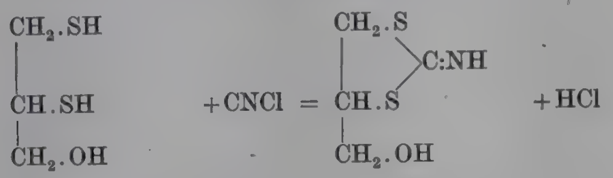
$$2\begin{array}{l}CH_2.SH\\ CH.OH\\ CH_2.SH\end{array} + 2CNCl = \begin{array}{l}CH_2.S—S.H_2C\\ CH.OH\ HO.HC\\ CH_2.S—S.H_2C\end{array} + 2HCN + 2HCl$$

or

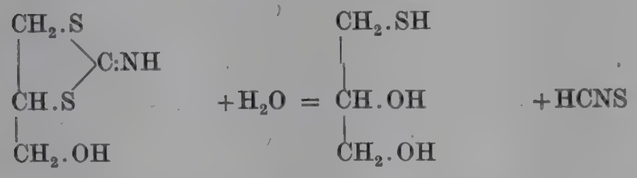


$$\begin{array}{l}CH_2.SH\\ CH.OH\\ CH_2.SH\end{array} + CNCl = \begin{array}{l}CH_2.S—\\ CH.OH\\ CH_2.S—\end{array} + HCN + HCl$$

Since 1 mol. of cyanogen chloride reacts with 1 mol. of BAL and both SH groups have been shown to be substituted, it is probable that the initial reaction product has a cyclic structure. Further, since thiocyanate is so easily split off, the compound must be unstable and since some indication of the reappearance of SH groups has been obtained, a reaction of the following type may take place:

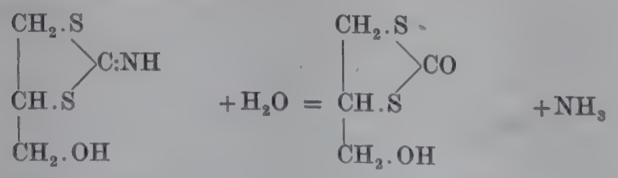


$$\begin{array}{l}CH_2.SH\\ CH.SH\\ CH_2.OH\end{array} + CNCl = \begin{array}{l}CH_2.S\\ \quad\ \ \rangle C{:}NH\\ CH.S\\ CH_2.OH\end{array} + HCl$$



$$\begin{array}{l}CH_2.S\\ \quad\ \ \rangle C{:}NH\\ CH.S\\ CH_2.OH\end{array} + H_2O = \begin{array}{l}CH_2.SH\\ CH.OH\\ CH_2.OH\end{array} + HCNS$$

The compound produced is stable in acid solution and bromination in acid solution without liberating thiocyanate produces no cyanogen bromide. There is also the possibility that an organic thiocyanate is formed, from which no cyanide or thiocyanate would be liberated with the mild conditions in the method. A further possibility is that the cyanogen chloride-BAL compound postulated might be hydrolyzed to produce ammonia, i.e.



$$\begin{array}{l}CH_2.S\\ \quad\ \ \rangle C{:}NH\\ CH.S\\ CH_2.OH\end{array} + H_2O = \begin{array}{l}CH_2.S\\ \quad\ \ \rangle CO\\ CH.S\\ CH_2.OH\end{array} + NH_3$$

The fact that only 0·8 mol. of thiocyanate is obtained from 1 mol. BAL may therefore be attributed to (*a*) impurity of the sample used for standardization and/or (*b*) other reactions taking place concurrently. Table 9 shows that from 1:2-dimercaptopropionic acid, 0·9 mol. thiocyanate is obtained/mol.; in view of the fact that this compound is a crystalline solid it is possible that it is the purest of the three dithiols examined. However, whether the variation in recovery of thiocyanate between 1:2-dimercaptopropionic acid and BAL is due to a difference in purity, or to the reaction taking a different course, is a very open question.

Application of the method to biological fluids

To determine BAL in blood, use has been made of the stability of the BAL-cyanogen chloride compound in acid solution and the fact that it is recoverable from blood after precipitation of protein. In view of the catalytic action of haemoglobin on the oxidation of BAL, cyanogen chloride has been added in isotonic solution and the protein precipitated by the method of Folin (1930). It should be noted that sodium sulphate must be used for the isotonic solution, and not sodium chloride, which acts as a peptizing agent for tungstic acid so that the centrifugates contain protein and colloidal tungstic acid. The addition of cyanogen chloride to blood produces some cyanide but no thiocyanate (Aldridge & Lovatt Evans, 1946). The cyanide is removed by aeration after protein precipitation. Cyanogen chloride is rapidly taken up by proteins in blood, and an excess must therefore be added to ensure a sufficiency for the BAL-cyanogen chloride reaction to go to completion.

In view of the instability of BAL in blood, indirect methods have been used for the determination of its recovery. Degradation curves have been prepared which have been extrapolated to zero time. (Fig. 1). By this means it has been shown that recoveries of 90–95 % may be expected both from whole blood and plasma. The degradation rate in plasma and serum seems to be higher than that in whole blood. Varying the amount of haemoglobin in the plasma or the addition of citrate and/or cyanide as metallic catalyst poisons had little effect on this rate (Figs. 2, 3). It is possible that this may be due to the incorporation of BAL into an oxidation-reduction system, which retards its oxidation in whole blood, while in plasma no such system is present. Thiocyanate blanks should always be carried out, though these are very low in whole blood because thiocyanate is absorbed by whole blood protein precipitates. Determination of 1:2-dithiols in normal whole blood has so far yielded negative results.

Much difficulty has been encountered in the determination of BAL in urine. The urine must be buffered to about pH 7 for the cyanogen chloride reaction and more bromine water must be added in order to leave an excess. Our examination of the recovery of BAL from various urines has given 100 % recovery only from rather dilute samples from human males; with these samples the ‘dithiol blank’ was zero. With other samples, in which the ‘dithiol blanks’ were high (Fig. 4), the recoveries were very low and the degradation curves were an extraordinary shape. On no occasion have we obtained 100 % recovery from female urine. In many cases we have experienced discolorations of the final colorimetric solution, showing a distinctly brownish shade when compared with those obtained with pure aqueous solutions of BAL.

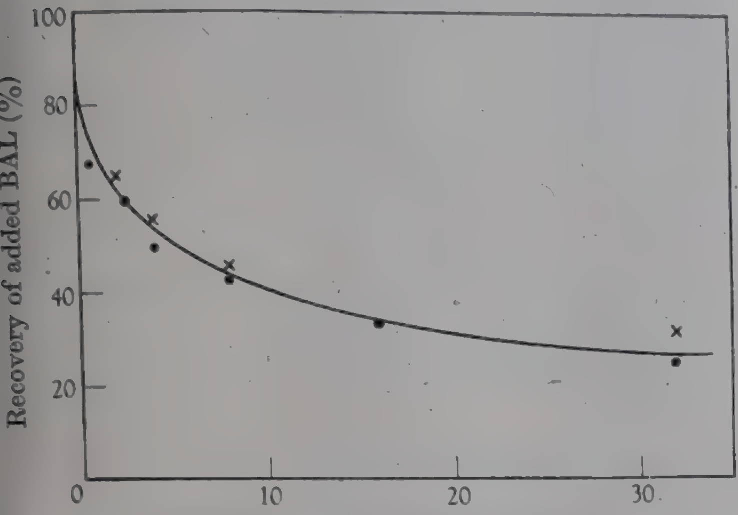


Fig. 1.

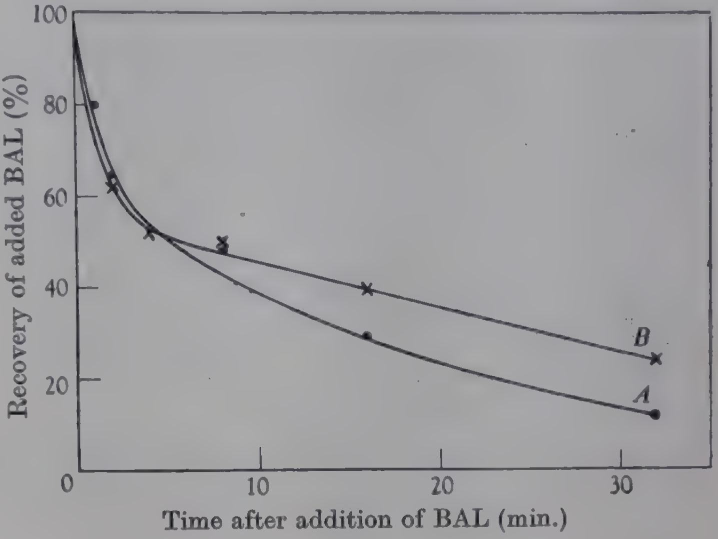


Fig. 2.

Fig. 1. Recovery of added BAL from whole blood. Two determinations plotted.

Fig. 2. Recovery of added BAL from serum. Curve *A*, cat serum (free from haemoglobin); curve *B*, same cat serum with added cyanide.

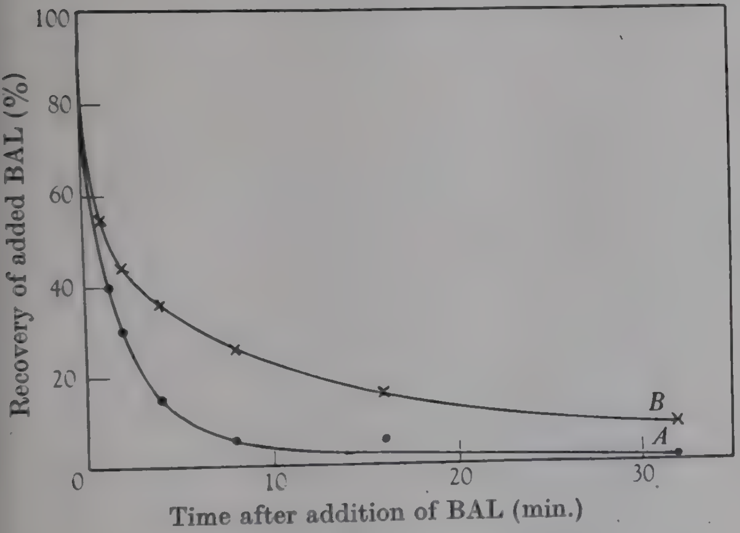


Fig. 3.

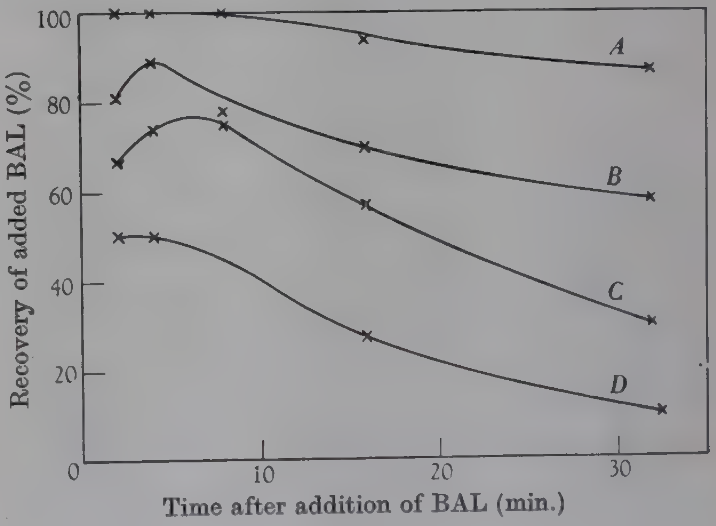


Fig. 4.

Fig. 3. Recovery of added BAL from plasma. Curve *A*, rabbit plasma from defibrinated blood; curve *B*, same rabbit plasma with added citrate.

Fig. 4. Recovery of added BAL from various samples of human urine. Curve *A*, male urine, 'dithiol blank' nil; curve *B*, female urine, 'dithiol blank' 3·6 μg./ml.; curve *C*, male urine, 'dithiol blank' 3·2 μg./ml.; curve *D*, female urine, 'dithiol blank' 11·3 μg./ml. (All 'dithiol blanks' calculated as BAL.)

Upon the recovery of added substances in biological fluids, a few general remarks are necessary. If the recovery of any substance is determined when added to biological media such as blood and urine, theoretical or lower than theoretical results are possible. There are two possible reasons for the low recovery (*a*) the analytical method is not estimating the substance owing to interference from other substances, or (*b*) the substance has been converted to some other derivative which is not determined. If (*b*) is true, the determination of the recovery of consecutive additions of the substance sometimes proves the point and the second addition gives a 100 % recovery. However, an experiment of this nature with BAL in urine gave 31 % recovery for the first addition and 46 % for the second.

SUMMARY

1. A new method for the determination of 1:2-dithiols is described, depending on the fact that cyanogen chloride reacts with 1:2-dithiols to give a compound which readily liberates thiocyanate in alkaline solution.

2. 1:3-Dimercaptopropanol and the oxidized form of BAL do not react. Of many thiol compounds, only glutathione, ergothioneine and thiolacetic acid interfere.

3. A method for the determination of BAL in blood is described, but a satisfactory method in the case of urine has not been found. The results obtained are discussed.

4. The mechanism of the reaction is discussed.

I wish to thank the Chief Scientist of the Ministry of Supply for permission to publish this work and Dr L. A. Stocken (Department of Biochemistry, Oxford) for many of the samples of thio compounds. I am also indebted to Mrs L. Currell and Miss A. E. Scott for their painstaking technical assistance; without their keenness this work would scarcely have been possible.

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* Information available on application to Ministry of Supply, London.

The Estimation of Urethane (Ethyl Carbamate) in Blood

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In connexion with the recent use of urethane in the treatment of leukaemia (Haddow & Sexton, 1946; Paterson, Ap Thomas, Haddow & Watkinson, 1946), the necessity arose for a method for estimating this substance in blood.

The very simplicity of the chemical structure, $NH_2.COOC_2H_5$, and its close resemblance to urea, indicated that attempts to estimate urethane by reactions based on the amino group were unlikely to be successful. Attention was therefore directed to the characteristic ester grouping. It was found that urethane is quantitatively hydrolyzed to ethanol, ammonia, and carbonate, by boiling with sodium hydroxide solution. The ethanol can be estimated by distillation into acid potassium dichromate solution followed by titration of excess dichromate with sodium thiosulphate after addition of potassium iodide.

METHOD

The estimation involves two stages:

(1) *Determination of volatile reducing substances in blood before alkaline hydrolysis.* Folin-Wu blood filtrate (10 ml.) is measured into a 100 ml. flask containing a few glass beads and connected with a Jackson's condenser which has its delivery tube extended downwards for about 15 cm. The delivery tube dips below the surface of a mixture of potassium dichromate and sulphuric acid (1 ml. of 0·1 N-$K_2Cr_2O_7$ and 5 ml. A.R. conc. H_2SO_4) which is contained in a tube of 20 ml. capacity. The tube containing the mixture is well cooled in ice. With no water cooling on the condenser, about 8 ml. of the flask's contents is distilled over into the oxidizing mixture. A glass stopper is sealed into the tube with a drop of conc. H_2SO_4 and the contents mixed and heated to 80° for 20 min. The tube is then cooled and the contents washed into a flask with about 100 ml. of water, 5 ml. of 5% KI solution are added and the liberated iodine titrated with 0·1 N-sodium thiosulphate.

The above determination usually yields a small blank value.

(2) *Determination of volatile reducing substances in blood after alkaline hydrolysis.* A clean flask is fitted to the condenser, and a mixture of 10 ml. of blood filtrate together with 5 ml. of 10 N-NaOH is refluxed for 15 min. The water supply to the condenser is then stopped and 8–10 ml. of the flask's contents distilled into acid dichromate mixture as in (1). The subsequent procedure is as described in (1) above.

The difference between the dichromate reduced in (1) and in (2) is a measure of the urethane content of the blood. 1 ml. of 0·1 N-dichromate is equivalent to 2·225 mg. of urethane.

RESULTS

As an illustration of the use of the method the results obtained on specimens of blood from two patients with leukaemia who received urethane orally are given.

Case 1. The patient had been receiving 1 g. of urethane daily for 50 days, the last dose being administered at 4.30 p.m. on the 50th day. On the 51st day, a sample of blood was drawn at 1 p.m. At 1.20 p.m. the patient ingested 2 g. of urethane. Blood samples were taken after this dose at 0·5, 2, 5·5 and 22·5 hr. The urethane content of these samples is shown in Table 1.

Table 1. *Blood urethane values of case* 1

Sample	Time (hr.)	Urethane (mg./100 ml.)
1	0	10
2	0·5	38
3	2	36
4	5·5	22
5	22·5	21

The urethane content of the first sample was produced by the preliminary treatment with urethane. Administration of 2 g. raised the level of urethane in the blood by 28 mg./100 ml. in the first 0·5 hr.

Case 2. Samples of blood were obtained before the patient had started urethane treatment. Urethane (3 g.) was then given daily for 14 days and the content of the blood measured at intervals. The results of these estimations are shown in Table 2.

Table 2. *Blood urethane values of case* 2

(Samples 1 and 2 were taken before starting urethane treatment.)

Sample	Day of treatment	Urethane (mg./100 ml.)
1	− 4	4
2	− 2	5
3	1	10
4	3	31
5	7	27
6	14	27

If the pretreatment blank values (samples 1 and 2) are taken as 5 mg./100 ml., then the daily ingestion of 3 g. of urethane maintained in this patient a blood level of about 22 mg./100 ml.

DISCUSSION

Theoretically, the method of estimation described above has the disadvantage that blood may contain compounds other than urethane that, on hydrolysis with alkali, may yield volatile oxidizable substances. In practice, however, it was found that normally this blank value was only about 3–5 mg./100 ml. of blood and did not interfere appreciably with the estimation. Care must be taken, however, that the patient is given no other drugs that may be so hydrolyzed, nor must the blood be preserved with such a substance. Heparin was found quite satisfactory as an anticoagulant for this purpose. It is essential that the blood samples should be as fresh as possible.

Attempts were made to shorten the procedure by carrying out the control and the urethane determinations on the same sample of filtrate. Blood filtrate was distilled down without collecting the distillate in order to remove volatile oxidizable materials. The volume was then restored by addition of water, 10N-NaOH added, and the mixture refluxed for 15 min. before distillation into dichromate. This method proved to be unreliable owing to the volatility of urethane. Up to 50 % of the total urethane was lost in the preliminary removal of the volatile substances. In view of this the standard procedure adopted was that described above in which the total urethane and the blank value are determined on separate samples of blood filtrate.

SUMMARY

1. A method is described for the estimation of urethane in the blood of patients undergoing therapy with this drug.

2. The urethane content of the blood can be maintained at a relatively high level for a prolonged period by the daily administration of 1–3 g. of urethane orally to an adult subject.

We are indebted to Prof. A. Haddow and Miss J. M. Watkinson for some of the blood specimens used in this work.

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Purification of Yeast Hexokinase and its Reaction with $\beta\beta'$-Dichlorodiethyl Sulphide*

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(*Received* 22 *April* 1947)

The enzyme theory of vesication, as developed by Peters and Dixon and their respective schools, provides that the primary attack of a vesicant is upon one or more specially sensitive enzymes whose destruction leads to the arrest of some essential metabolic processes of the cell. The vesicant action of compounds such as lewisite and mustard gas ($\beta\beta'$-dichlorodiethyl sulphide, described hereafter as H) have thus been studied in relation to their effect upon respiring tissue slices or *brei*, and also upon isolated enzyme systems concerned with intermediary carbohydrate metabolism. In this laboratory, we were more immediately concerned with the role of mustard gas in the inhibition of skin glycolysis, and attention became focused upon hexokinase when there was found to be a correlation between the fall of skin glycolysis, the onset of skin damage, the lowered hexokinase activity of extracts of such skin, and the sensitivity of hexokinase to mustard gas *in vitro* (Dixon, Needham & van Heyningen, 1942; see also review by Dixon & Needham, 1946).

At this stage it was thought desirable to study the action of the vesicant upon the pure enzyme, which could only be obtained in reasonable amount from yeast. The limited objective was to find whether a sensitive enzyme under comparable conditions of treatment reacts more readily than do other proteins whose amino-acid composition is approximately known; the further objective, not yet achieved, was to establish the nature of the sensitive group, assumed to be the active enzyme centre.

Part 1 of the present paper describes the purification, crystallization and properties of yeast hexokinase, and Part 2 describes the action of SH poisons and vesicants. The effect of SH poisons (including lachrymators) is only summarily reported, since the results with the pure enzyme confirm the earlier work of van Heyningen (1942) using crude hexokinase. The inhibition by H on the other hand, is described in some detail, and embraces two types of experiment; first, the relation between extent of inhibition and inhibitor concentration under varying conditions, and secondly, the quantitative assay of the number of vesicant groups combined with the enzyme at the point of complete, or almost complete, inactivation. With the small amount of crystalline enzyme at our disposal, this work was only possible by use of mustard gas containing radioactive sulphur (H^*), and by the collaboration of Drs Boursnell, Francis and Wormall, who kindly determined the radioactive S content (S^*) of hexokinase thus inactivated. Under these 'mild' conditions of inhibition, when the enzyme is subjected to a solution of vesicant (as distinct from the more drastic treatment with droplets of liquid vesicant, cf. Herriott, Anson & Northrop, 1946), the uptake of mustard gas by hexokinase was compared with that of other proteins of well defined amino-acid composition and containing a representative number of reactive side-chain groups, viz. ovalbumin and fibrinogen. Moreover, a comparison was made of the amount of vesicant combined with ovalbumin after both mild and drastic treatment, in order to obtain some estimate of the maximal number of vesicant groups which can combine with a protein as compared with the number necessary for enzymic inactivation.

PART 1. PURIFICATION AND CRYSTALLIZATION OF YEAST HEXOKINASE

Yeast hexokinase was partially purified by Meyerhof (1927), van Heyningen (1942) and Dixon & van Heyningen (unpublished), the latter employing adsorption on calcium phosphate. The present authors then continued the research at a stage when American workers had also taken up the problem of vesication. The crystallization of hexokinase was first reported by Cori, Colowick, Berger & Slein (1942), using methods involving ethanol precipitations, and later by Northrop, Kunitz & McDonald (1942) (see also Kunitz & McDonald, 1945–6), using mainly ammonium sulphate fractionation. Our method was developed independently and differs from those of the American workers, but in the last stage of purification we made use of the Kunitz-McDonald newly reported and elegant method for the final crystallization. The activity and crystalline

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* This work formed part of the research programme carried out for the Ministry of Supply by an Extra-Mural Research Team under the direction of Dr M. Dixon, and was reported to the Ministry of Supply (Bailey & Webb, 1944*a*).

form of our preparation were similar to those of the crystals which Dr Northrop kindly sent us. It is not clear whether the 'hexagonal plates' obtained by Cori and co-workers represent the same or a different crystalline form, but after crystallization by the Kunitz-McDonald method they are similar to their and our crystals (Berger, Slein, Colowick & Cori, 1945–6).

Activity estimations

The decrease in easily hydrolyzable phosphate during the incubation of the enzyme with adenosinetriphosphate (ATP), glucose and Mg^{++} was determined by the Fiske-Subarrow method, after hydrolyzing the digest with N-HCl for 7 min. at 100°. The method in principle is essentially that given by van Heyningen (1942). The incubation mixture contained 0·5 ml. 0·1 M-MgCl$_2$, 0·6 ml. M-glucose, enzyme diluted to 0·5 ml., and 0·1 M-veronal-acetate buffer pH 7·9 to a total volume of 4·5 ml. Sodium-ATP solution (0·5 ml.) containing *c.* 1·2 mg. 7 min. P was added in the cold, and the mixture placed in the bath at 38°. The amount of enzyme taken was sufficiently small to give an approx. linear breakdown for 7–8 min. Samples of 1 ml. were removed at 1, 3, 5 and 7 min. and pipetted directly into 0·12 ml. 10 N-HCl. After immersion in a boiling water bath for 7 min., each sample was washed into a 50 ml. flask, mixed with Fiske-Subarrow reagents, diluted to volume, and the orthophosphate determined colorimetrically against a standard containing 0·2 mg. P in 50 ml.

One hexokinase unit (HK unit) was defined as the amount of enzyme which transfers 1 mg. 7 min. P from ATP to glucose in 1 hr. under the conditions of the above test, the initial linear portion of the curve 7 min. P against time being used.

Purification of hexokinase

Stage 1. *Autolysis of baker's yeast.* Preliminary experiments showed that autolysis of yeast with toluene and water takes place in two stages. No hexokinase activity is given in the standard test by a suspension of fresh baker's yeast, but yeast treated with toluene for 30 min. at 38° shows a maximal activity soon after mixing with water if the whole suspension is used. At this point, very little of the enzyme is in true solution but is merely accessible to the substrate. On more prolonged incubation of the autolysate, the enzyme gradually passes into solution, and eventually the activity of the supernatant liquid equals that of the whole suspension. Prolonged autolysis at 38°, however, causes considerable destruction of enzyme (up to 50% in 2 days) before extraction is complete (Exps. 1*a*, *b*, Table 1). If, after the preliminary toluene treatment for a few hours at 38°, the autolysis is allowed to proceed at room temperature, destruction losses are smaller, especially in presence of 1% glucose, but the activity of the supernatant liquid usually attained its maximum only after several days. The general effect of glucose, whilst conferring stability, was to retard autolysis (Exps. 2*a*, *b*).

It was then found that addition of SH compounds (cysteine or neutralized Na$_2$S) had the combined effect of stabilization, accelerating hydrolysis and increasing the final yield of liberated hexokinase (Exps. 3*a*–4*b*). (These results suggest that English baker's yeast is much more

Table 1. *Liberation of 'fixed' hexokinase from yeast under varying autolysis conditions*

	Conditions of autolysis			Hexokinase content (units/ml.)	
Exp.*	Temp. (° C.)	Additions	Duration (hr.)	Whole suspension	Supernatant liquid
1*a*	38	None	18	34	14
			42	14	Nil
1*b*	38	1% glucose	18	21	8
			42	10	Nil
2*a*	20–25	None	18	62	18
			42	40	43
2*b*	20–25	1% glucose	18	45	13
			42	45	23
			66	45	35
			90	43	48
3*a*	20–25	1% glucose	42	38	26
			96	43	31
3*b*	20–25	0·1 M-cysteine	42	56	42
			96	53	53
3*c*	20–25	0·1 M-NaSH	42	42	46
			96	51	42
4*a*	20–25	None	18	51	12
			42	(39)	26
			66	50	34
4*b*	20–25	0·1 M-NaSH	18	49	41
			42	66	58

* Different yeast samples behave differently under standard conditions of autolysis. Since various samples were used, comparison can only be made between results of each individual numbered experiment.

resistant to autolysis than the yeasts used by the American workers. Similar differences between Delft and Manchester yeasts have been noted by Bach, Dixon & Zerfas (1942, 1946) in the preparation of lactic dehydrogenase. Prof. F. Dickens (private communication) has experienced similar difficulties in extracting hexokinase from Distillers Company Ltd. baker's yeast. The earlier work of van Heyningen (1942) suggests that the role of SH compounds in the stabilization of the enzyme may be the prevention of oxidative destruction; the acceleration of autolysis, on the other hand, may be due to their activating effect on proteolytic enzymes which liberate hexokinase from the particulate components of the cell.

The autolysis was carried out in batches as follows: 28 lb. (12·7 kg.) of baker's yeast (Encore) were crumbled, mixed with 760 ml. toluene and maintained at 38° for 3–5 hr. To the liquid mass were added 12 l. of cold tap water, together with 100 g. $Na_2S.9H_2O$ and enough conc. HCl to restore the pH to 6–6·5 (*c.* 50 ml. 10 N-HCl). The mixture was centrifuged after standing for 42 hr. at 20–25°, and the supernatant liquid treated with solid $(NH_4)_2SO_4$ to 75% saturation. The protein was filtered off under suction and stored as a moist filter cake at 0°. Altogether, 185 lb. (84 kg.) of yeast were worked up in this way.

Stage 2. *Dialysis.* The collected filter cakes were dissolved in an equal volume of water. An attempt to fractionate with $(NH_4)_2SO_4$ was abandoned, as the precipitate could not be spun or filtered. The liquid (9·4 l. containing 4,350,000 HK units) was dialyzed against distilled water at 1° in 40 cellophan sacs. After changing the water daily, the $(NH_4)_2SO_4$ concentration fell after 7 days to 0·55 g./l.

Dialysate: 12·8 l.; 3,300,000 HK units; 56 units/mg. N.

Stage 3. *Adsorption.* (Here, as in subsequent adsorptions, small scale experiments were first carried out. A known volume of calcium phosphate gel was added to the enzyme solution and spun down; after determining the enzyme activity of the supernatant liquid, a further portion of gel was added and the whole process repeated until the hexokinase was entirely adsorbed. In the main solution this procedure was shortened by adding just the amount of gel to take down impurities, and after centrifuging, a subsequent portion to adsorb most of the enzyme.) The dialysate (pH 6) was stirred with 5 l. of calcium phosphate gel (4·4% dry wt.), which was spun off and discarded. The enzyme was adsorbed on a further 5 l. of gel, and after centrifuging, the precipitate was eluted six times with Na_2SO_4 solution (100 g. anhydrous salt/l.); 30 g. glucose were added to the eluates to stabilize the enzyme.

Eluates: 7·13 l.; 1,750,000 HK units; 113 units/mg. N.

Stage 4. *Ammonium sulphate fractionation.* Solid $(NH_4)_2SO_4$ was added to give 55% saturation. (Here again, the conditions for fractional salting out were determined in small-scale experiments.) The precipitate was filtered off under gravity and rejected. The salt concentration was then increased to 75% saturation, the precipitate filtered off, dissolved in 1% glucose solution and dialyzed against several changes of 1% glucose at 0° for 3 days.

Dialysate: 885 ml.; 860,000 HK units; 264 units/mg. N.

Stage 5. *Adsorption.* The solution was treated with 88 ml. calcium phosphate gel, spun, and the precipitate rejected. Another 550 ml. of gel were added, spun off, and the precipitate eluted with Na_2SO_4-glucose solution (100 g. anhydrous sodium sulphate, 10 g. glucose/l.).

Eluates: 925 ml.; 670,000 HK units; 550 units/mg. N.

Stage 6. Attempts to increase the activity by fractional salting-out, or to crystallize the enzyme were unsuccessful. Eventually all fractions were combined, salted out with $(NH_4)_2SO_4$ (85% saturation), filtered under suction, taken up in a small volume of water and dialyzed against 0·1 M-acetate buffer at pH 5·9 and containing 1% glucose. About 210,000 HK units at 300 units/mg. N resulted from these unsuccessful trials. In future preparations, these losses would be avoided by passing on to the next step, thereby increasing the eventual yield threefold.

Stage 7. *Ethanol precipitation.* Absolute ethanol at −10° was added to the cooled solution (98 ml.) to give a final concentration of 34% (v/v). The precipitate was centrifuged down at 0° and discarded. The ethanol content of the supernatant liquid was raised to 53% (v/v), the precipitate centrifuged down, dispersed immediately in 1% glucose, and insoluble material separated by filtration through paper pulp. All operations were carried out at or near 0°.

Solution: 172 ml.; 115,000 HK units; 400 units/mg. N.

Stage 8. *Crystallization.* Although at stage 7 the preparation was less pure than at stage 5, it yielded readily to the crystallization procedure used by the Northrop school. It seems very probable that the ethanol solutions removed lipid material which prevents crystallization. The solution from stage 7 was salted out in 85% saturated $(NH_4)_2SO_4$ solution, and the protein obtained as a filter cake. It was dissolved in a small quantity of 0·1 M-phosphate buffer pH 7, saturated ammonium sulphate was added to a faint turbidity, and the liquid allowed to drain through a filter at 0°. After a week, crystals (A) appeared in the filtrate. The residue on the paper was again dissolved and treated as above, and yielded a second crop (B). The crystals were washed in 75% saturated $(NH_4)_2SO_4$ solution, and dissolved for activity tests in 3% Na_2SO_4 containing 1% glucose.

Crystals A: 4 mg. protein N; 6200 HK units; 1530 units/mg. N.

Crystals B: 13 mg. protein N; 15,600 HK units; 1200 units/mg. N.

From the mother liquors, further small crops were obtained which eventually were combined with the main crops and recrystallized.

The final product, reproduced in Pl. 1, contained 13 mg. protein N (i.e. 83 mg. protein) and 40,000 HK units at 2500–3000 units/mg. N. The crystals were stored moist in saturated ammonium sulphate solution at 0°.

Comparison of the activity of the crystals with the preparations of Cori and of Northrop. Northrop *et al.* (1942) reported an activity of 1440 Cori units/mg. protein for five times recrystallized hexokinase, i.e. 9230 Cori units/mg. protein N. Assuming a temperature coefficient for the enzyme reaction of $Q_{10}=2$, calculation shows that 1 Cori unit (defined as the amount of enzyme catalyzing the formation of acid equivalent to 1 µl. CO_2/min. at 25°) is equal to 0·205 of our HK unit (defined as the amount causing transfer of 1 mg. P/hr. at 38°). Using this conversion factor, Northrop's reported activity is equal to 1890 HK units/mg. N.

Cori, Colowick, Berger & Slein (1943), however, later reported an activity of 2100 Cori units/mg. protein for a sample of hexokinase obtained from orthrop. Converted to our units, this corresponds o an activity of 2750 HK units/mg. N. In agreenent with this value, a sample of Northrop's rystalline hexokinase, kindly made available to us y Prof. Peters, gave an activity of 2850 HK nits/mg. protein N under our test conditions. These igures are similar to those for our own preparation.

Summarization of purification data. The quanitative data on the purification process are brought ogether in Table 2. The early steps of alternate alting out and adsorption give a much more rapid purification than the repeated salt fractionation adopted by Northrop and co-workers. In the one case, the activity is roughly doubled at each stage, in the other, a 25 % increase in purity was achieved.

The pure, recrystallized preparation represents a recovery of c. 1·2 % enzyme and a 53-fold purification on the dialysate of stage 2. With our present procedure, the yield could possibly be increased to 3·6 % by passing from stage 5 to 7.

Table 2. *Purification of yeast hexokinase*

Stage	Volume (l.)	Total activity* (hexokinase units)	Purity (units/mg. N)	Total N* of fraction (g.)
2. Filter cake from 84 kg. yeast	10·9	$4·7\times10^6$	—	—
Same after dialysis	12·8	$3·3\times10^6$	56	59·0
3. Adsorption and elution	7·13	$1·75\times10^6$	113	15·5
4. $(NH_4)_2SO_4$ fractionation	0·885	860,000	264	3·25
5. Adsorption and elution	0·925	670,000	550	1·22
6. Unsuccessful fractionation; fractions combined	0·215	210,000	300	0·70
7. Ethanol precipitation	0·172	115,000	400	0·30
8. Crystals A	—	6,200	1530	0·004
Crystals B	—	15,600	1200	0·013
Recrystallized (A, B and other crops)	—	40,000	2500–3000	0·013

* Activity and N (micro-Kjeldahl) determined on dialyzed material; corrections for traces of NH_3-N applied after distilling undigested sample at pH 9·5 in the micro-Kjeldahl apparatus.

Properties of hexokinase

An extensive examination of the properties of hexokinase was not undertaken, since the material was required for combination with ββ'-dichlorodiethyl sulphide containing radioactive S. Moreover, the general properties of the enzyme (metal activation, stability, pH optimum, substitution of other sugar substrates for glucose) had already been determined for a partially purified preparation by van Heyningen (1942). Similar investigations have also been made by Berger *et al.* (1945–6) and by Kunitz & McDonald (1945–6) on the crystalline enzyme.

Our own preparation dissolved in water to give a colourless solution. Except in high salt concentration or in presence of glucose the enzyme was rapidly inactivated (without visible precipitation) above pH 7: 5 min. at 38° and pH 7·9 sufficed to inactivate completely, whilst at the same pH and 20°, 90 % of the activity was lost in 2·5 hr. No evidence of protection from spontaneous inactivation was observed with glycine or with cyanide, although the latter salt was stated by van Heyningen (1942) to act as stabilizer.

The enzyme was inactive in absence of Mg ions, and these could not be replaced by Ca^{++}. The activity in presence of 5×10^{-3}M-Mg^{++} was not affected by the addition of an equal concentration of Ca^{++}; the Ca : Mg antagonism which is so pronounced in the case of the Mg-activated yeast pyrophosphatase (Bailey & Webb, 1944*b*) is thus absent in the case of hexokinase. It would be of interest to discover whether such antagonism as is found in yeast pyrophosphatase and myosin adenosinetriphosphatase is general for those enzymes producing free phosphate, but absent in the phosphate-transferring enzymes (phosphokinases). The proteins ovalbumin and insulin, found by Cori *et al.* (1943) to produce activation of the enzyme, were quite ineffective with our preparation. As compared with the theoretical value of 50 %, about 43 % of the easily hydrolyzable P of ATP was transferred to glucose in presence of the enzyme in accordance with the generally accepted view that hexokinase catalyzes the reaction:

$$\text{Adenosinetriphosphate} + \text{glucose} \rightleftharpoons \text{Adenosinediphosphate} + \text{glucose-6-phosphate}$$

Physical constants for the enzyme have been obtained by Kunitz & McDonald (1945–6). The protein was found to be electrophoretically pure, and to give a solubility independent of the amount of solid phase. Cataphoretic studies gave an isoelectric point between pH 4·5–4·8, and sedimentation-diffusion a molecular weight of 96,600.

PART 2. INHIBITION OF HEXOKINASE BY SH POISONS AND BY $\beta\beta'$-DICHLORODIETHYL SULPHIDE (*H*)

SH *poisons including lachrymators*

All SH poisons, including lachrymators and the vesicant lewisite, were most powerful inhibitors, and much more effective than iodoacetate. Of inhibitors unrelated to vesicants and lachrymators, 0·04 M-NaF gave a 46 % inhibition under the standard test conditions.

In Table 3 are listed the inhibitions obtained with varying concentrations of SH poisons. Except where otherwise stated, the poisons were incubated with enzyme in veronal-acetate buffer pH 7·9 containing $1{\cdot}1\times10^{-3}$ M-MgCl$_2$ and M/73 glucose. Additional glucose (to raise the concentration to that used in the standard activity assay) was added with the ATP after this preliminary incubation. The marked inhibition which occurs at very low concentration in the case of chloroacetophenone, bromobenzyl cyanide, ethyl iodoacetate and lewisite, known from the work of Mackworth (1941, 1947) to be SH-combining reagents, is in general agreement with the results of van Heyningen (1942) carried out with an impure preparation, and support her conclusion that the presence of SH groups in the hexokinase molecule is essential for its activity.

Table 3. *Effect of* SH *poisons on hexokinase*

Inhibitor	Concentration of inhibitor	Conditions of preliminary incubation		Inhibition (%)
		Time (min.)	Temp. (° C.)	
Chloroacetophenone	M/880	15	38	33
	M/880	15	38	50
	M/880	5	16	Nil
Bromobenzyl cyanide	M/800	5	38	100
Ethyl iodoacetate	M/800	5	38	49
Na iodoacetate	M/63	5	38	100
	M/200	5	38	20
Lewisite (in M/8 glucose)	M/1000	15	38	100
	M/2000	15	38	70
	M/2500	15	38	43
	M/4000	15	38	20

The inactivation of crystalline hexokinase by radioactive H (*H**)

It was our aim to treat the enzyme with *H* under the mildest conditions consistent with a high degree of inactivation, so as to reduce to a minimum any general attack which might conceivably accompany the inactivation process, although playing no essential part in its causation. Preliminary experiments were carried out with non-radioactive *H* in order to determine these conditions. The inherent instability of the enzyme, however, creates special difficulties. van Heyningen (1942) reported an 80 % inactivation by $2{\cdot}5\times10^{-3}$ M-*H* (initial concentration) in 0·014 M-veronal-acetate buffer at pH 7·8 and 38° in presence of 0·007 M-glucose. Under these conditions, a considerable amount of spontaneous inactivation occurred in the control without *H*, and although this was allowed for in the calculation of percentage inhibition, it is essential for our present purpose that any inactivation other than that due to *H* should be eliminated as far as possible. Without this provision, any comparison between extent of inactivation and the number of *H* groups combining is meaningless.

Unfortunately, the conditions for greatest sensitivity to *H* are also those of least stability. Enzymes in general seem to become more sensitive to *H* as the pH is raised (see Dixon (1943) and Table 4), but with hexokinase, pH values above 7 cannot be employed at temperatures higher than 20° because of the spontaneous inactivation which then occurs. The enzyme is more stable at lower temperatures, but these necessitate a long incubation with *H* to allow of complete reaction. Again, the presence of glucose, whilst effecting stabilization, reduces the sensitivity of the enzyme to *H* (Dixon, 1943). With reasonably small amounts of glucose (*c.* 0·01 M) the inactivation by *H* could be measured without serious spontaneous inactivation, either by incubation at 38° in buffer at pH 5·5, or by treatment at room temperature in buffer at pH 7·4–7·9. A few results with crystalline hexokinase are shown in Table 4; similar results were obtained with a crude yeast autolysate and with a partially purified preparation. In each case, a solution of *H* in ethylene glycol monoethyl ether was added to a dilution of enzyme sufficient for one test, to give a solution of concentration $2{\cdot}5\times10^{-3}$ M. The activities after incubation were measured at pH 7·5 or 7·9 and compared with a control treated similarly with *H*-solvent in absence of *H*.

Table 4. *Inhibition of crystalline hexokinase by H*

		Time of incubation with	Composition of incubation mixture				
pH	Temp. (° C.)	$2{\cdot}5\times10^{-3}$ M-H (min.)	Buffer	Glucose (M)	Mg^{++} (M)	Enzyme N/ml. (μg.)	Inhibition (%)
7·5	16	135	0·08 M-veronal-acetate	0·011	0	2·4	25
7·9	20	15	0·09 M-veronal-acetate	0·0001	0·013	1·2	44
5·5	38	15	0·1 M-acetate	0·0056	0	2·8	13
5·5	38	15	0·1 M-acetate	0	0	1·4	10

From Table 4 it will be seen that hexokinase is comparatively insensitive to H at pH 5·5, even in the absence of glucose. At the higher pH values and lower temperature, the inactivation is much more marked, and the protection by glucose is in evidence. It was clear from these results that it was not possible both to obtain full sensitivity to H and freedom from spontaneous inactivation, and that a compromise must be sought. It was finally decided to sacrifice sensitivity to some extent in order to reduce the spontaneous inactivation to a reasonably small value, and to attain the necessary degree of inhibition by treating the enzyme several times in succession with small amounts of H.

Treatment of hexokinase with radioactive H. The compromise conditions for obtaining a reasonable sensitivity to H and low spontaneous inactivation were realized by treating with H at room temperature at pH 7·4 in presence of M/180 glucose. Additions of radioactive H in an organic solvent were made at intervals of about 45 min. until a sufficient degree of inactivation had been obtained, each addition in the concentration of $2{\cdot}5\times10^{-3}$ M. A control without H gave a measure of the spontaneous inactivation. With the amount of crystalline enzyme available (80 mg.) only two experiments could be carried out. These are described below.

Exp. 1. Crystalline hexokinase containing 5·1 mg. protein N was dissolved in 5 ml. 0·1 M-phosphate buffer pH 7·4 containing M/180 glucose. For activity measurements, 0·01 ml. samples were pipetted into 1 ml. of ice-cold water and 0·1–0·3 ml. of this solution was used for each test. At various intervals, shown in Table 5, 0·05 ml. of 0·25 M-H in ethylene glycol monoethyl ether was added, until the activity fell to a fairly low value. No strictly parallel control to determine the extent of spontaneous inactivation was made in this experiment, but the first diluted sample, taken before H addition, was incubated under the conditions of the main experiment and used as a diluted control; this procedure is open to criticism since dilution may have the effect of decreasing stability, the control thus giving too high a value for spontaneous inactivation. In Exp. 2, therefore, this defect was remedied by incubating a control under the same conditions as those of the experimental solution.

Exp. 2. The enzyme (6·8 mg. protein N) was dissolved in 5·5 ml. 0·1 M-phosphate buffer pH 7·4 containing M/180 glucose. A 5 ml. sample was used for H treatment and 0·5 ml. incubated similarly as a control without H, the same concentration of H solvent (in this case ethanol) being added. The results are shown in Table 5.

In both experiments, the H-treated solutions remained clear and gave no visible evidence of denaturation.

Table 5. *Treatment of crystalline hexokinase with radioactive H*

(0·05 ml. 0·25 M-H^* added to final concentration of $2{\cdot}5\times10^{-3}$ M at times marked →.)

Exp. no.	Time of activity determination (hr.)	Activity of H^*-treated enzyme as % of original (HK units/ml.)	Activity of control as % of original (HK units/ml.)
1	→ 0	100	100
	→ 0·75	95	—
	→ 1·75	—	—
	→ 2·5	40	—
	3·25	23	77*
2	→ 0	100	100
	→ 0·5	—	—
	→ 1·25	—	—
	→ 1·5	—	—
	2·0	31	97
	→ 3·0	—	—
	3·75	29	91

* 'Diluted' control, see text.

Determination of combined H. The H-treated hexokinase from each experiment was precipitated with an equal volume of ethanol and allowed to settle overnight. It was washed thrice with 50 % (v/v) ethanol and twice with absolute ethanol. After Soxhlet extraction with acetone and subsequently with ether to remove any H^* and thiodiglycol containing S^*, it was dried over $CaCl_2$. Protein N was determined by the micro-Kjeldahl method on a sample of the dried powder after removing non-protein N with 5 % trichloroacetic acid, and the remainder was used for S^* determinations. These latter were carried out by Drs Boursnell and Francis (cf. Boursnell, Francis & Wormall, 1946) using the Geiger counter method as adapted by Banks (1946). From the N and S^* determinations the number of H molecules combining with each molecule of enzyme was calculated, taking the N content of ash-free and moisture-free hexokinase as 15·6 %, and the molecular weight as 97,000 (Kunitz & McDonald, 1945–6). The results are shown in Table 6. For reasons given above, the control in Exp. 2 has been used in the calculations for Exp. 1, rather than the 'diluted' control. Since both experiments were carried out under very comparable conditions, the rates of spontaneous inactivation could have differed only slightly. The number of *H* groups/molecule of enzyme inactivated by *H* is calculated on the assumption that the degree of inactivation is proportional to the *H* groups combined. In both experiments this number corresponds to 6–7 groups/molecule enzyme, or, assuming the average amino-acid residue weight to be comparable to that of a polar protein such as fibrinogen, i.e. 114, to the combination of less than 1 *H* group in 100 amino-acid residues. By comparison, the extent of substitution when the enzyme is stirred with liquid *H* (at pH 5·5–6·0) can be gauged from the work of Herriott *et al.* (1946) who found that 30–35 *H* groups combined with the enzyme. This latter figure suggests a general attack upon the enzyme, whilst our own much lower figure is scarcely more than might be expected from a specific combination of *H* with the active enzyme centres.

Table 6. *Estimation of H^* groups combined with hexokinase*

	Exp. 1	Exp. 2
Protein-N content of powder (%)	10·62	11·5
Wt. taken for S^* determination (mg.)	35·5	(*a*) 20·75 (*b*) 17·45
S^* found (as mg. H^*)	0·169	(*a*) 0·105 (*b*) 0·094
Molecules H^*/molecule hexokinase	4·3	(*a*) 4·2 (*b*) 4·4
		Mean 4·3
Total enzyme inactivation (%)	77	71
Inactivation in control (%)	(9)	9
Inactivation due to *H* (%)	68	62
Estimated no. *H* groups/molecule hexokinase for 100 % inactivation by *H*	6·3	7·0

The reaction of other soluble proteins with H

An enzyme may be particularly sensitive to *H* either because (*a*) it contains specially reactive groups, and is capable of substitution under conditions which are without effect on insensitive enzymes and presumably on proteins in general, or because (*b*) although the reactivity may be similar to that of other proteins, the reacting groups are specially connected with enzyme activity. To distinguish between these possibilities in the case of hexokinase it seemed desirable to compare the behaviour of other proteins, using the same mild conditions of *H* treatment. The proteins selected were thrice recrystallized ovalbumin, and six times precipitated ox fibrinogen.

Conditions of H treatment. The proteins were dissolved in phosphate buffer at pH 7·5 to give a concentration of 7–8 mg./ml. A solution of *H* in ethanol (0·25 M) was added in five successive portions at intervals of 0·75 hr., each portion being 1/100 of the volume of the original solution. The temperature was maintained at 20°. Under these conditions, the *H* was in clear solution, and throughout the reaction the sols remained free of precipitated protein. After treatment the sols were dialyzed, precipitated with an equal volume of acetone after adjusting to pH 5·5, washed four times with acetone, four times with ethanol, and finally with ether. Samples of untreated protein were similarly precipitated and washed, and finally dried over $CaCl_2$ for N and S determinations.

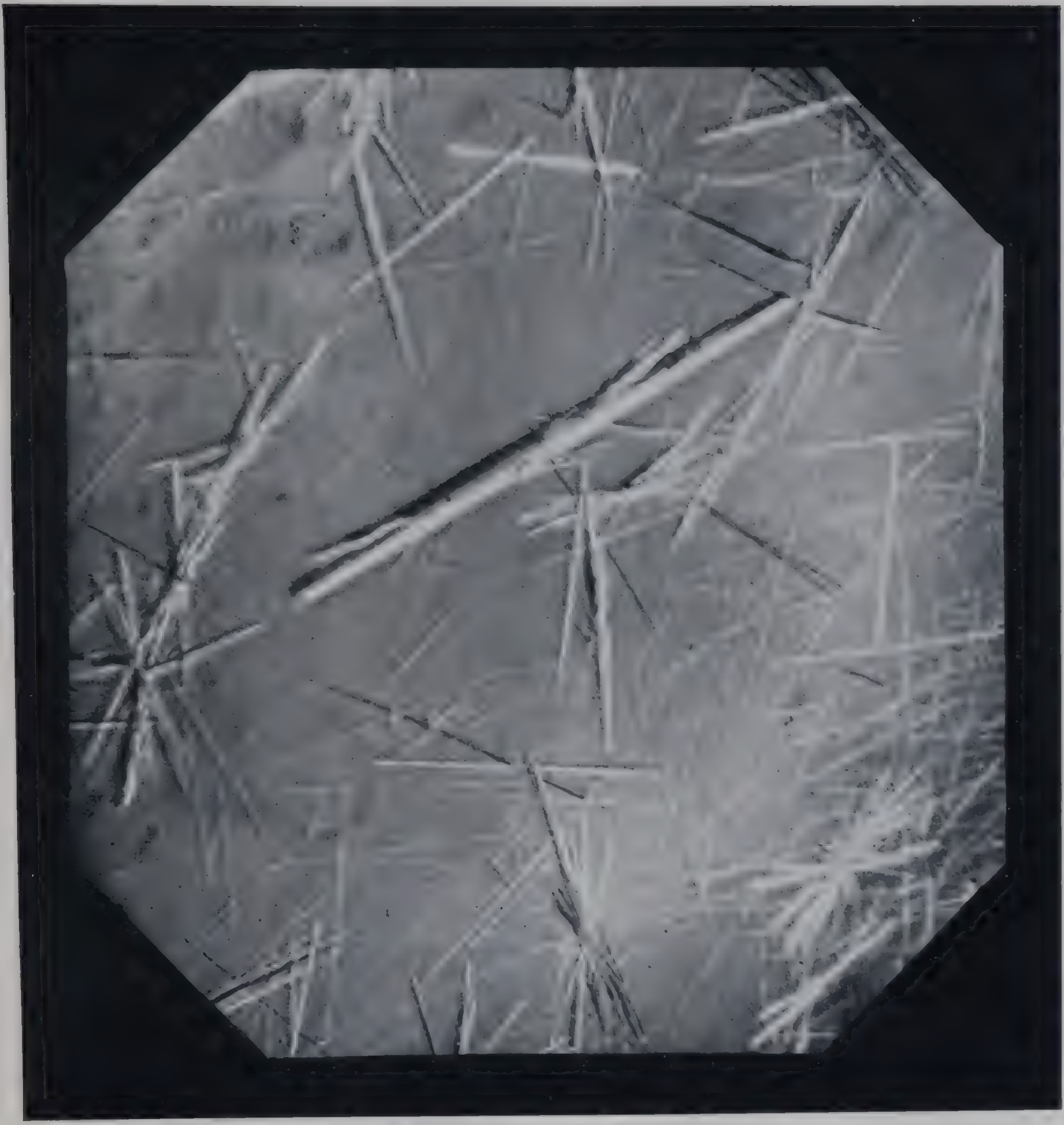
For comparison, ovalbumin was also treated with liquid *H*. The protein (4·5 g.) in 80 ml. water was stirred with 1 ml. of liquid *H*, dispersed in fine droplets, for 10 hr. at 30°; solid $NaHCO_3$ was added at intervals to maintain the pH at 7. The *H*-treated sol was then decanted from residual *H*, dialyzed against water, adjusted to pH 5·5, precipitated with 10 vol. acetone, and Soxhlet-extracted for 6 hr. each with acetone, ethanol and ether.

Sulphur determinations. The HNO_3-$KClO_3$-$Cu(NO_3)_2$ oxidation generally employed for the determination of S in *H*-treated proteins (e.g. Boursnell *et al.* 1946) is lengthy and sometimes violent. The alternative method of alkali fusion gives a very complete oxidation of the S-amino-acids of proteins but with *H*-treated products, it is open to the objection that combined *H* groups may be split off and lost in the form of volatile sulphides or their oxidation products. This difficulty has been overcome by mixing the finely-ground protein (0·3 g.) with a large excess of fusion mixture (8 g. of 1 : 1 Na_2CO_3 and Na_2O_2) and heating in a nickel crucible on a hot-plate at 140–160°. After 0·5–1 hr. the mixture gently fuses, each protein particle burning at red heat in the oxidizing medium. The melt is then heated to redness for 3–4 min., cooled, and sulphate estimated gravimetrically using glass-distilled water. The reagent blank amounted to 1 mg. $BaSO_4$. The S content of crystalline *H* sulphoxide determined by this method was 18·4% (theory, 18·3%).

The combination of H with ovalbumin and fibrinogen. The number of *H* groups in combination with 10^5 g. protein (chosen as a basis for comparison with hexokinase, which has a molecular weight of nearly 10^5) was calculated from the expression

$$\frac{1000\,N^0}{32}\left(\frac{S^H}{N^H}-\frac{S'}{N'}\right),$$

where S' and N' are the S and N contents of the untreated protein (not necessarily dry); S^H and N^H are the values for the *H*-treated sample, and N^0 the true N percentage of the original ash-free protein dried at 105°. The results are summarized in Table 7. Despite the fact that the analytical methods used cannot determine the extent of substitution with any great precision when only small differences of S content are measured, it is nevertheless clear that the number of *H* groups combining under the 'mild' treatment are comparable with those in hexokinase; such differences as exist can largely be explained on



Crystalline yeast hexokinase (× 135).

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Table 7. *Treatment of ovalbumin and fibrinogen with H*

Protein	Conditions of treatment	Untreated (dried at 105°) N (%)	Untreated (dried at 105°) S (%)	*H*-treated (air-dried) N (%)	*H*-treated (air-dried) S (%)	Mol. *H*/10^5 g. protein
Fibrinogen*	0·025 M-$NaHCO_3$	16·7	1·16	(*a*) 15·85	1·32	7·2
	0·06 M-phosphate			(*b*) 15·9	1·34	7·8
	0·15 M-NaCl				1·28	5·8
	(*H* in solution)					Mean 7·0
Ovalbumin	0·1 M-phosphate	15·5	1·56	14·0	1·55	4·9
	(*H* in solution)				1·59	6·3
						Mean 5·6
Ovalbumin†	$NaHCO_3$	15·5	1·56	12·95	2·19	33
	(liquid *H*)				2·25	35
						Mean 34
Hexokinase (see Table 6)	(*H* in solution)	—	—	—	—	4·3

(*a*) and (*b*) represent separate experiments, not duplicate analyses.

* After *H*-treatment, the fibrinogen sol in presence of purified thrombin still clots; the clotting time is only slightly delayed, but the clot is at first of loose texture and opalescent, and becomes more rigid on standing.

† *H*-treated ovalbumin after 24 hr. at pH 11 and room temperature contains 14·8% N and 1·95% S (air-dried), corresponding to 15 mol. *H*/10^5 g. protein.

the basis of differences in buffer competition. From these experiments, therefore, it appears that the sensitivity of hexokinase to *H* is not due to any special reactivity of the molecule as a whole, but rather to the dependence of enzyme activity upon groups which are relatively easily substituted by *H*.

As against the six *H* groups combined with 10^5 g. ovalbumin after mild treatment, some 34 are taken up when treated with liquid *H*. These figures run parallel with those already discussed for hexokinase under the two comparable sets of conditions. When allowed to stand at room temperature at pH 11 for 24 hr., 19 of the 34 groups were found to be alkali-labile, indicating that 56% of the groups substituted are probably in ester linkage with carboxyl groups. The residual (alkali-stable) groups may involve amino groups, though according to Herriott *et al.* (1946) these are not substituted in the case of ovalbumin. None of the groups was hydrolyzed by a partially purified liver esterase preparation, although this enzyme is relatively non-specific, hydrolyzing almost all esters.

It may be noted that the extent of *H* substitution in the case of ovalbumin treated with liquid *H* corresponds to one *H* group/26 amino-acid residues. It is remarkable, therefore, that the protein evinces no sign of denaturation and remains soluble both at pH 7 and 5, but, like the native form, is readily heat-coagulated at the latter pH. This is in contrast to the behaviour of nucleoproteins which become insoluble after reaction with *H* (Berenblum, 1940). As recent work (Auerbach & Robson, 1946) suggests that nuclear changes are manifest in *H*-damaged tissue, this difference of behaviour may be of great importance to a complete understanding of the toxicology of mustard gas.

SUMMARY

1. The purification and crystallization of hexokinase from baker's yeast are described. The method was developed independently of those of the Cori and Northrop schools, but the procedure of the latter for the final step of crystallization has been followed. The crystals are of the same form and activity as those of Northrop.

2. The addition of cysteine or NaSH to autolyzed baker's yeast accelerates the liberation of hexokinase from the particulate components of the cell and retards inactivation.

3. As found by van Heyningen (1942) for impure hexokinase, the pure enzyme is stabilized by glucose and is activated by Mg ions. This activation is not antagonized by Ca ions.

4. The enzyme is inhibited by SH poisons (including lachrymators), suggesting that hexokinase is an SH enzyme. These results confirm those of van Heyningen (1942) using impure hexokinase.

5. Since vesicant action is accompanied by *in vivo* inactivation of hexokinase (Dixon *et al.* 1942), the uptake of vesicant by yeast hexokinase has been studied, using ββ′-dichlorodiethyl sulphide containing radioactive S. By comparison with other proteins treated with vesicant under similar conditions, it is concluded that the sensitivity of hexokinase to mustard gas is not due to any special reactivity, but to the dependence of enzyme activity on groups which are relatively easily substituted.

The authors are grateful to Mr B. R. Slater for his help during the initial stages of purification, and to the Chief Scientist of the Ministry of Supply, for permission to publish this paper.

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The Metabolism of the Lungs from Normal Mice and from Mice Exposed to Phosgene

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The respiration and glycolysis of the lungs of normal mice, and of mice previously exposed to phosgene, have been measured in order to determine if the oedema following exposure is preceded by inhibition of the carbohydrate metabolism. This was done in the hope that knowledge of the nature of the changes might lead to a rational therapy of phosgene poisoning.

EXPERIMENTAL

The measurements were made with the Warburg apparatus at 38°. The lungs were suspended in Ringer-phosphate in the presence of O_2 for respiration measurements and in Ringer-bicarbonate in 95% N_2/5% CO_2 for measurement of the glycolysis. To minimize injury to the lung, the whole lobes from mice of about 20 g. wt. were used for the measurements. Lungs of larger animals would have necessitated the use of minced or sliced tissue. Manometric readings were taken at intervals over a 2 hr. period. The tissue in each experiment was weighed (*a*) before the manometric measurements were made, (*b*) in the wet state at the end of the manometric measurements and (*c*) after drying to constant wt. at 100°. The metabolic quotients (Q) are expressed in μl./mg. dry wt./hr.

RESULTS

Comparison of metabolism of whole lobes and cut lung tissue from normal mice

The lungs of freshly killed normal mice were removed and washed with Ringer solution. The lobes of one lung were weighed, and placed whole in a Warburg vessel, while the lobes of the other lung were minced into fine pieces with scissors before being placed in a second vessel. Some values for metabolism of whole and minced tissue are given in Table 1. The mean values for Q_{O_2} with glucose are 7·3 for whole tissue, 7·4 for minced tissue (eight determinations of each type). Similar experiments have shown that the respiration in the absence of glucose, and the glycolysis in the presence of glucose, are not seriously changed by mincing of the tissue. These results indicate that measurements made on whole lobes of small mice are not affected by the rates of diffusion of O_2 or CO_2 in the tissue and give a true picture of the activity of the enzymes within the tissue. The slight increase, which is found in the minced tissue, is probably due to the loss of material in the Ringer solution so that the dry weights of tissue are really somewhat low. The values for respiration are similar to those obtained for rat lung by Laser (1932), but the values for glycolysis are higher than those found with rat lung.

Table 1. *The effect of mincing on the metabolism of normal mouse lung*

	Wt. of tissue (mg.)			Metabolism		
Nature of tissue	Before exp. (wet)	Dry	Increase in wet wt. during exp. (%)	Respiration with glucose Q_{O_2}	Respiration without glucose Q_{O_2}	Glycolysis of glucose $Q_G^{N_2}$
Whole lobes	70	14	52	6·4	—	—
Minced tissue	79	14	133	7·8	—	—
Whole lobes	95	16·2	10	6·9	—	—
Minced tissue	119	18·6	2	6·5	—	—
Whole lobes	65	13·2	48	7·5	—	—
Minced tissue	71	13	12	7·7	—	—
Whole lobes	68	10·6	43	—	7·5	—
Minced tissue	100	17	21	—	8·3	—
Whole lobes	84	17	50	—	6·1	—
Minced tissue	88	15	Nil	—	7·1	—
Whole lobes	74	15	19	—	—	3·0
Minced tissue	69	13	17	—	—	3·8
Whole lobes	85	16	47	—	—	4·0
Minced tissue	120	20	15	—	—	5·4
Whole lobes	82	14	26	—	—	3·5
Minced tissue	89	17	26	—	—	3·6

Measurements of normal lung tissue showed that the respiration is not increased by addition of glucose to the medium. Mean values obtained for Q_{O_2} with glucose were 7·3 (individual values 6·3, 6·3, 7·8, 7·9 and 8·6), and Q_{O_2} without glucose, 7·6 (individual values 6·3, 6·4, 6·5, 6·7, 7·0, 7·2, 7·3, 7·5, 8·0 and 9·1). Similar figures were obtained for the respiration in the presence of DL-alanine, viz. Q_{O_2} without substrate 5·8–7·0, and Q_{O_2} with 0·025 M-alanine 6·4–6·8. These results indicate that, if the utilization of glucose or alanine were specifically inhibited by phosgene, such inhibition might not reduce the respiration. The glycolysis values ($Q_G^{N_2}$) found were 2·7, 2·8, 2·9, 2·9, 3·1, 3·1, 3·4 and 3·4, having a mean of 3·0.

In all cases in which measurements were made with lungs from normal mice, the tissue increased in weight over the experimental period. The increase in weight varied from 0 to 133 % of the original weight; it is due in part to replacement of air in the lung by Ringer solution, and also to swelling of the tissue.

Effect of exposure to phosgene

Mice were exposed to air containing phosgene in a chamber as described by Boyland, McDonald & Rumens (1946), the concentrations being checked analytically. The results obtained on the lungs of such exposed mice are set out in Table 2. It is clear that there is no significant change in the respiration or glycolysis of the lung tissue following exposure to lethal doses of phosgene.

Most of the lungs of mice exposed to phosgene increased in weight during the measurements, often to the same extent as did normal lungs. In one group of mice which were exposed to a high dosage ($Ct = 4080$ mg. min./m.^{3}, where C = concentration of phosgene in mg./m.^{3} and t = time in min.), 19 hr. before examination, the wet weights of the lungs decreased during the experiment. This was presumably due to oedema fluid, and possibly other material being washed away with Ringer solution while the manometric measurements were being made. It is possible that a reversal of tissue swelling occurred in the Ringer solution.

The change in weight of the lung following exposure could be seen by comparing the weight of the wet lung with the total body weight of the mice. Some estimations of nucleic acid phosphorus were carried out by the method of Berenblum, Chain & Heatley (1939). The data (Table 3) show how the

Table 2. *Metabolism of lungs from mice exposed to phosgene*

Details of exposure (dosages (Ct) in mg. min./m.³)	Wt. of tissue (mg.) Before exp. (wet)	Wt. of tissue (mg.) Dry	Change in wet wt. during exp. (%)	Q_{O_2} with glucose	Q_{O_2} without glucose	Glycolysis of glucose $Q_G^{N_2}$
$Ct=2100$; $t=8$ min. (less than 1 hr. before measurement)	70	12·8	—	7·1	—	—
	103	20·2	—	7·7	—	—
	61	8·8	—	9·2	—	—
	124	23·0	+24	6·0	—	—
	92	17·0	—	—	6·4	—
	44	8·2	—	—	6·4	—
	122	23·6	+32	—	6·2	—
	122	22·0	+47	—	6·2	—
	150	29·6	—	—	—	2·5
	195	39·6	—	—	—	2·5
	161	32·0	+49	—	—	3·0
	165	31·4	+36	—	—	2·6
$Ct=5300$; $t=8$ min. (less than 1 hr. before measurement)	108	16·6	+15	7·1	—	—
	116	17·4	+10	7·8	—	—
	66	12·4	+38	7·6	—	—
	71	11·8	+36	7·7	—	—
	73	10·6	Nil	—	5·9	—
	135	24·9	+42	—	5·9	—
	98	16·0	+19	—	6·6	—
	101	19·0	+18	—	6·6	—
	133	33·4	+56	—	—	3·2
	176	30·8	+ 6	—	—	4·1
	200	35·0	+26	—	—	3·3
	158	29·0	+12	—	—	2·8
$Ct=4200$; $t=2$ min. (3 hr. before measurement)	61	10·0	+41	6·3	—	—
	69	10·0	+20	5·1	—	—
	126	22·4	+29	—	7·0	—
	136	20·4	− 6	—	6·9	—
	177	32·4	+18	—	—	2·9
	207	31·8	Nil	—	—	2·6
$Ct=4080$; $t=8$ min. (19 hr. before measurement)	71·3	9·0	−11	5·7	—	—
	155	16·0	−32	5·2	—	—
	84	10·4	− 6	—	6·1	—
	161	16·6	−32	—	4·3	—
	146	18·6	−19	—	—	2·95
	116	12·9	−32	—	—	3·5
$Ct=4200$; $t=2$ min. (27 hr. before measurement)	34	5·4	−24	7·6	—	—
	30	4·4	−17	7·7	—	—
	78	13·4	−26	—	7·7	—
	54	8·8	−13	—	7·0	—
	104	19·4	−31	—	—	3·0
	86	25·2	−17	—	—	2·1

weight of lung tissue increases following exposure to phosgene causing a relative decrease in the nucleic acid phosphorus of the lungs.

DISCUSSION

The experiments reveal no great change in the metabolism of the lung following exposure to phosgene. If the oedema of the lung were due to the poisoning of enzymes concerned in carbohydrate metabolism some change should have been observed. Thus, although it may be possible that vesication of the skin by mustard gas is due to poisoning of hexokinase, it seems very improbable that poisoning of this enzyme or of any of the other enzymes concerned with glycolysis in the lung, occurs on exposure to phosgene.

SUMMARY

1. The lungs of young adult mice are sufficiently small to allow of measurement of metabolism without mincing. Mean values of 7·3 for Q_{O_2} and 3·0 for $Q_G^{N_2}$ were found.

2. No great changes in the metabolism of the lung were observed on exposure of mice to phosgene, and it therefore appears unlikely that the oedema of the lung is due to poisoning of enzymes concerned with glycolysis or respiration in the lung.

We are indebted to the Chief Scientist of the Ministry of Supply for permission to publish these results.

Table 3. *Weight of lung and nucleic acid phosphorus in the lungs of normal and phosgene-poisoned mice*

Details of mouse		Wet wt. of lung (mg.)	Wt. of lung as % of body wt.	Nucleic acid P of lung	
Exposure	Wt. (g.)			(As % of lung wt.)	(As % of body wt.)
ne	19	133	0·705	0·115	0·00081
	23	156	0·678	0·109	0·00073
	21	152	0·722	0·114	0·00082
	19·5	148	0·759	0·108	0·00076
	22	141	0·640	0·107	0·00069
	20	161	0·806	0·108	0·00087
	21·8	146	0·650	0·112	0·00075
	22·5	196	0·871	0·110	0·00086
	18	100	0·556	0·098	0·00055
	19·5	135	0·693	0·108	0·00073
	20·5	143	0·698	0·111	0·00077
	19·5	122	0·626	0·115	0·00072
osgene $Ct = 2480$ (4½ hr. efore estimation)	20·5	312	1·52	0·062	0·00094
	22	301	1·37	0·069	0·00094
	23	310	1·35	0·064	0·00086
	19·5	314	1·61	0·058	0·00094
	19	296	1·56	0·059	0·00092
	21	320	1·52	0·064	0·00098
hosgene $Ct = 2240$ (22 hr. efore)	19	277	1·46	0·051	0·00075
	20	213	1·06	0·081	0·00086
	21	269	1·28	0·069	0·00088
	20	305	1·52	0·051	0·00078
	19	164	0·864	0·087	0·00075
	20·5	185	0·903	0·087	0·00078

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The Electrophoretic Analysis of Protein Extracts from Striated Rabbit Muscle

2. DENATURATION IN ACETATE BUFFERS

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In a previous paper (Jacob, 1947) some interrelations between the classical muscle proteins (myogen, myoalbumin, myosin, globulin *X*) and the electrophoretic components of various extracts of striated rabbit muscle were established. In order to complete the characterization of components, the analysis of one of these muscle extracts in acetate buffers of decreasing pH is described here. This is necessary since the isoelectric points of several components lie in the pH range covered by acetate buffers, and in an acid medium, most of the muscle proteins are denatured without flocculating (Finn, 1932; Weber, 1934; Bate Smith, 1937). These new observations are of interest in the study of denaturation processes.

EXPERIMENTAL

The electrophoretic technique and the procedures for the extraction and preparation of muscle extracts have been described in a previous paper (Jacob, 1947). The extraction solution used here contained 0·048 M-Na_2HPO_4 and 0·006 M-NaH_2PO_4 ($pH = 7·7$, $\mu = 0·15$); the dialysis solutions, which also served as supernatant in the electrophoretic experiments, contained 0·1 M-sodium acetate, 0·05 M-NaCl and a variable amount of acetic acid. Their pH varied from 3·62 to 5·92, with ionic strength constant at $\mu=0{\cdot}15$.

The extracts were dialyzed for 24–70 hr., and some protein was found to precipitate. When the extract was centrifuged after 3 days' dialysis, the volume of precipitate was greater the lower the pH of dialysis. When the extract was centrifuged several times during dialysis, it was possible to collect a precipitate during the first 36–60 hr., in the pH range 5·0–5·9; whereas, at any lower pH, after the 8–24 hr. precipitates had been removed, no further precipitation occurred.

The total amount of precipitate was larger and its formation more rapid the lower the pH. The dialysis time was sufficient to avoid further precipitation during electrophoresis, though at pH 5·9 failures were frequent. The dialyzed extracts became less turbid as the pH decreased, and below pH 5·0 they were almost limpid.

When dialyzed extracts were left at room temperature, a further precipitate appeared; the quantity and rapidity of formation of this increased with the fall of pH, thus illustrating the progressive instability of the extracts. Between pH 3·9 and 3·6, the extracts were extremely viscous and had a tendency to form a thixotropic gel, even when kept at 1°.

As in the preceding paper, pH measurements were made at room temperature. To calculate the pH at 0°, the values were corrected by 0·025 pH unit, according to Harned & Ehlers (1932).

The relative amounts of the different components were obtained from the peak areas after correcting for significant superimposed δ and ϵ effects. Depending on the pH, there were two possibilities: (1) the δ and ϵ effects were superimposed on a protein peak located at the extremity of the pattern (here this peak is *h*); (2) the δ and ϵ effects were superimposed on one or more peaks located in the centre of the pattern (here these effects shift progressively from *l* to *h* as the pH decreases).

The first case is often recorded in the literature, and the values obtained from the descending pattern are usually adopted. By this means, the amount obtained for the extreme peak represents in fact the total of the amounts which can be attributed to that peak and to the ϵ effect, which is generally neglected. In our experiments, when the ϵ effect is dissociated from the protein peaks, it represents on the average 4% of the total surface of the diagram. Consequently, the values obtained from the descending pattern were corrected, by subtracting from the apparent amount of the extreme peak the amount due to the ϵ effect (4% of the total surface), and sharing this amount between the peaks, in proportion to their relative magnitude (this is in conformity with the theoretical developments of Longsworth, 1942).

When the δ and ϵ effects are superimposed on one or more median peaks, it is sufficient to consider that each of the patterns, the anode and cathode, contains ascending, descending and median peaks. The first step is to determine, on both diagrams, the relative amounts of the descending peaks. The difference between 100% and the total of these relative amounts represents the percentage to be attributed to the median peak and to the ϵ effect. It is then easy to eliminate the ϵ effect by the same procedure as in the first case.

It would have been a great advantage, if it had been possible, to correct the ascending values, in order to measure asymmetries depending on causes other than δ and ϵ effects. Unfortunately, the precision of the measurements was considerably reduced by the poor separation of one ascending peak at any pH higher than 5·0, and by the excessive sharpness of the gradient of another ascending peak at lower pH values.

Finally, it was impossible to establish whether the relative amounts of the different peaks altered during the course of electrophoresis.

RESULTS

Description of the patterns obtained in acetate buffers

At pH 5·9–5·8, the patterns obtained in acetate buffers differ only slightly from those obtained (Jacob, 1947) at neighbouring pH values in phosphate buffers. The separation into three main groups again occurs (Fig. 1). The first group consists of the peak *n*, which separates early, and of the two components *l* and *m*, which only appear after prolonged experiments at lower ionic strength ($\mu=0{\cdot}10$). The second group contains its usual components k_1, k_2 and *j*. Between groups II and III lies a small quantity of protein which is not easily divided between these two groups. For convenience it was called *sp* (space). The third group consists of a single peak, unimpaired by any turbidity. Probably the turbid protein *i* has vanished from it, and this peak represents the component *h* to which it approximates in amount.

The relative concentrations of the components are not widely different, whether the dialysis buffer is acetate or phosphate (Table 1).

As the pH decreases, the patterns change progressively (Fig. 1). The relative area of peak *n* increases regularly from 18% (pH 5·9) to 99% (pH 3·64) (Table 1, col. *C*). This increase in concentration is not so regular when expressed as mg. N/ml. because of non-systematic variations (up to 20% at least) in the total protein-N concentration of the dialyzed extracts. If these errors are taken into account, the change referred to the amount of N is also regular (Fig. 2). The concentration of peak *n* rises progressively to 1·2, 2·0, 2·4, 3·5 mg. N/ml., values which are much higher than the concentration of the substance *n* in extracts dialyzed against phosphate buffer (0·72–0·90 mg. N/ml.; Jacob, 1947). This peak thus represents not only the substance *n*, but also other proteins which behaved as separate entities in phosphate buffer. Consequently another designation *C* was adopted for this peak.

The shape of *C* differs in the anode (descending) and cathode (ascending) patterns. Up to pH 4·0, the characteristics of this asymmetry are classical (Tiselius & Horsfall, 1939; Longsworth & MacInnes, 1940). The ascending boundary is sharp and very homogeneous; the descending boundary is wide, and its 'buffer' side is steeper than its 'protein' side; this becomes more typical and almost excessive, as the pH decreases. Below pH 4·0, the descending

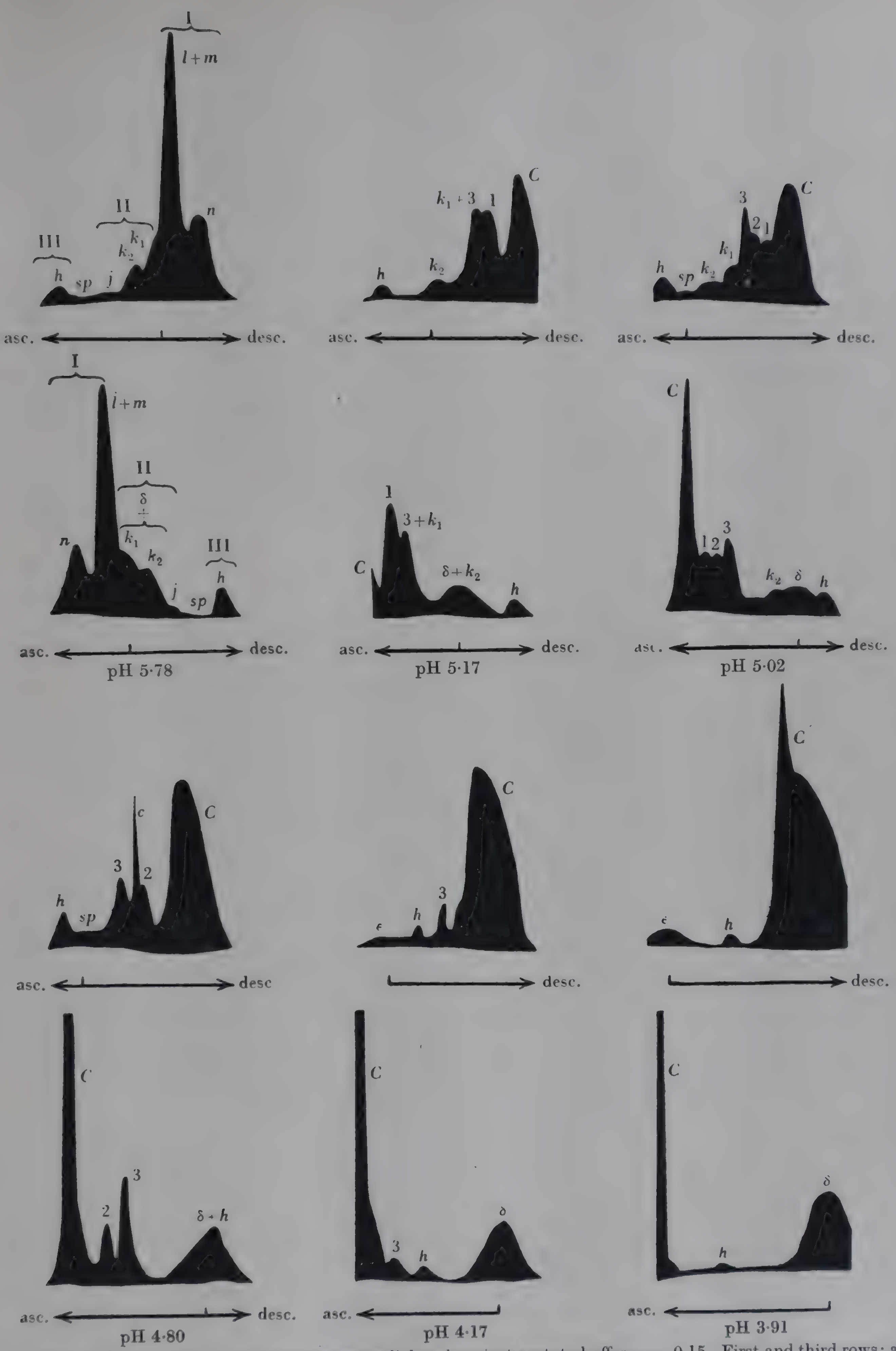

Fig. 1. Electrophoretic patterns of muscle extracts dialyzed against acetate buffers, μ = 0·15. First and third rows: anodic patterns; second and fourth rows: cathodic patterns; asc., ascending part; desc., descending part from the origin *O*. Details of runs were as follows:

pH	V./cm.	Sec. of electrophoresis
5·78	4·07	18,840
5·17	4·38	19,080
5·02	4·00	20,460
4·80	4·33	20,160
4·17	4·00	16,920
3·91	4·27	18,300

Table 1. *Composition of muscle extracts dialyzed in acetate buffers*

No.	pH	Protein-N (mg./ml.)	Relative amounts of the components from corrected descending values (%)					
			C	*l*+*m*	k_1	k_2+j	*sp*	*h*
1	5·92	1·80*	19·5	54·5	8·0†	9·0†	3·0	6·0
P‡	5·79	—	19·5	55·0	18·0		2·0	5·5
2	5·78	—	23·5	[55·0]	[7·5]	6·0†	2·5	5·5
3	5·62	—	33·0	51·0	5·0†	4·5†	2·5	4·0
4	5·61	3·61	35·5	48·0	5·0†	5·5†	2·0	4·0
5	5·45	2·52	34·5	50·0	5·0†	4·0†	2·5†	4·0
6	5·36	3·02	40·5	43·0	6·0	4·5†	2·0†	4·0
7	5·33	3·05	39·5	43·5	6·0	3·5†	1·5†	6·0
8	5·22	3·87	44·5	39·5	6·0	4·0†	2·0†	4·0
9	5·17	2·69	46·0	[40·5]	[4·5]	3·0†	2·0†	4·0
10	5·05	4·32	48·0	40·0	4·0	2·0†	2·0†	4·0
11	5·02	2·86	50·0	32·5	7·0	2·5†	1·5†	6·5
12	4·91	2·71	57·5	34·5			3·0†	5·0
13	4·85	4·00	62·0	31·0			2·0†	5·0†
14	4·80	3·51	66·5	27·0			2·0†	4·5†
15	4·69	—	69·5	24·0			2·5	4·0†
16	4·45	—	77·5	18·5			—	4·0†
17	4·34	3·66	81·0	14·5			1·0	3·5
18	4·17	3·80	[86·5]	[9·5]			—	4·0
19	3·91	3·92	98·0	—			—	2·0
20	3·64	—	99·0	—			—	1·0

* Artificially reduced value, not accounted for in Fig. 2.

† Indicates the peaks to which the ϵ effect was added.

P‡ Experiment with phosphate buffer.

[ ] Indicates the peaks whose separation was not satisfactory.

boundary splits in two and then several streaks form, whereas the ascending boundary apparently becomes more homogeneous. This suggests that the progressive spreading of the anode curve from pH 5·0 to 4·0 is a prelude to its cleavage at all lower pH values.

The peak common to *l* and *m* remains homogeneous down to pH 5·30 and its relative amount decreases from 54·5 to 43 %. From pH 5·2 to 4·45, this boundary undergoes several successive scissions. The patterns become complex and are characterized by asymmetries which even involve the number of peaks. Simultaneously, the total amount corresponding to these peaks falls as low as 18·5 %.

Between pH 5·20 and 5·05, *l*+*m* divides into two components whose relative amounts differ in the anode and cathode compartments (peak 1 (the faster) and peak 3); there is nothing to facilitate identification of these peaks with the components *l* and *m*.

In the neighbourhood of pH 5, an additional peak (peak 2) appears between 1 and 3; 1 becomes gradually smaller and simultaneously migrates closer to *C* and, mainly on cathodic patterns, its final inclusion in *C* can clearly be followed.

From pH 4·9 downwards, the asymmetry between the two patterns becomes more complex. In the anode pattern a component (*c*) appears whose gradient is relatively very sharp, and no corresponding peak forms in the cathode pattern.

Below pH 4·45, the group is simplified by the fusion with *C* of the residue of the streaks developed from the cleavage of boundary *l*+*m*.

The peaks of group II disappear progressively. Component k_1 separates less from peak 3, mainly on the cathode diagram, and below pH 5·00 it merges into peak 3. Component k_2 is sharply defined down to pH 4·9. It decreases in amount from pH 5·30 to 4·9 and at pH 4·8, no trace of it remains. Component *j* is marked by a slight inflexion on the side of k_2, which gets less and less distinguishable. Component *h* is, of all components, the one which undergoes the least alteration. Down to *p*H 4·1, its quantity measured from the descending boundary does not diminish, and it shows no peculiar characteristic. Below pH 4·0, it decreases, but at pH 3·64, it is still evident as a small inflexion. It is the only one among the peaks occurring at pH 5·9 which does not completely disappear, or is not completely absorbed by *C*.

Down to pH 4·85, the diagrams are mixed (Jacob, 1947), i.e. both the anode and cathode diagrams include a δ and an ϵ effect. The most obvious of these, the cathode δ effect, shifts progressively from *l* to *sp* between pH 5·9 and 4·9; it then reaches streak *h*, which it lifts, as far as pH 4·4, and then separates completely. The ϵ effect shows very clearly in the anode diagrams below pH 4·4.

Finally, the slight turbidity is no longer associated with groups II or III as in the phosphate experiments, but migrates with group I from pH 5·6 downwards.

In short, with the progressive fall of pH, the proteins tend to migrate as a single boundary (*C*).

The other components, with the exception of h, finally disappear either because they are incorporated in C, or because they precipitate in the course of the preliminary dialysis (which is probably the case for j and k_2). Before disappearing, some components undergo alterations (in the case of $l+m$ and k_2).

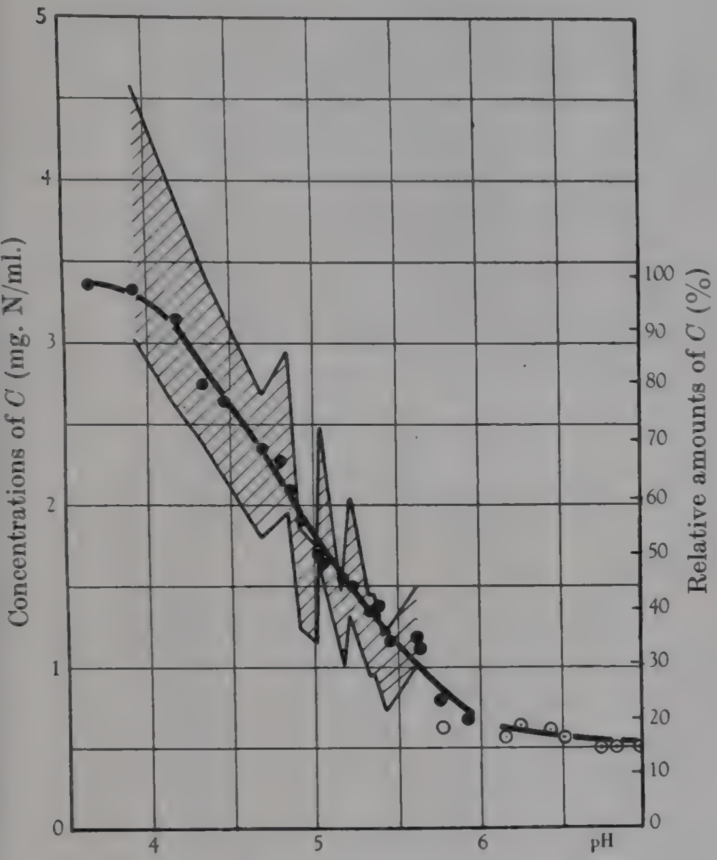

Fig. 2. Concentrations (in mg. N/ml., left scale) and relative amounts (in %, right scale) of boundary C as a function of the pH. The relation of the two scales was established with 100 % as an average of the total amounts of protein N in the dialyzed extracts (3·4 mg. N/ml.). The streaked area is obtained by joining the points corresponding to the concentrations increased and lowered by 20 %. ● Relative amounts of boundary C (acetate buffer); ○ relative amounts of boundary n (phosphate buffer – values previously published (Jacob, 1947)). The corresponding pH values have since been corrected according to Bates & Acree (1945).

Among more than thirty experiments, a single one—executed at pH 5·15—gave aberrant patterns in which the normal components maintained their individuality; the reason for this divergence could not be found.

Complex formation between components

The progressive incorporation of the protein components of the extracts into one electrophoretic peak can have two interpretations: either (a) the mobilities of different components become so similar in some zones of pH that no electrophoretic separation is possible under the conditions of the experiment; (the resulting peak has no special implication) or (b) a complex is formed between two or more proteins, and migrates with a characteristic mobility, distinct from those of the original proteins.

The first possibility seems likely, since the mobility curves of several components finally coincide with the curve of C (Figs. 3, 4), but this cannot wholly account for the behaviour of C, since before the fusions occur the amount of C increases considerably (from 18 % at pH 5·9 to 57·5 % at pH 4·90). Further, the contribution of each protein does not account quantitatively for the increase of C (for instance, from pH 4·90 to 4·40, the amount of C rises from 57·5 to 77·5 %, whereas the amount of peak 1 is, at the most, 7–8 %); finally, the increase of C is not irregular but almost continuous (Fig. 2). Probably C is primarily a complex composed of n and a portion of the other components.

Interactions are likely to occur between complex C and several other components, at all pH values lower than 4·9. Indeed, we notice the existence on the anode pattern of a peak (c), which does not appear on the cathode pattern.

A plausible interpretation is the following (according to Longsworth & MacInnes, 1942). On the anode (descending) side, the components 1, 2 and 3 migrate in a medium devoid of C. They have a tendency to re-form the complex C, but in the absence of n which has also left the medium, the aggregation product migrates more slowly than C and is represented by peak c. The mobility of c is not widely different from the average mobilities of 1, 2 and 3 at pH 4·8 and 4·9, and of the average mobilities of 2 and 3 at all lower pH's when 1 has been completely absorbed by C. This does not occur on the ascending side where components 1, 2 and 3 migrate in a medium containing complex C. On the contrary, one would expect complex C to dissociate when it migrates alone; this probably occurs, but the errors involved in the area measurements of the ascending peaks make it difficult to establish.

Further, important asymmetries occur between the ascending and descending mobilities. It is, in general, very difficult to estimate the part to be attributed to the 'classical' asymmetry, and to the 'interaction' asymmetry, but this is not the case for peak h below its isoelectric point. For this component, the 'classical' asymmetry is almost negligible owing to its low concentration (Jacob, 1947). This is true down to pH 4·67, but below this pH, i.e. when h begins to form part of the complex, the asymmetry becomes considerable (Fig. 4). Undoubtedly interactions occur which cannot otherwise be detected, owing to the difficulty of measuring exactly a peak of such small area.

It should be mentioned that dilution of the extract causes a diminution in the amount of complex C (for instance, the extract no. 9, diluted once with acetate buffer of an identical pH contains only 39 % of C instead of 46 % and likewise, the extract no. 14, diluted in the same manner contains only 55 % instead of 66 %). This agrees with some observations of Longsworth, Cannan & MacInnes (1940),

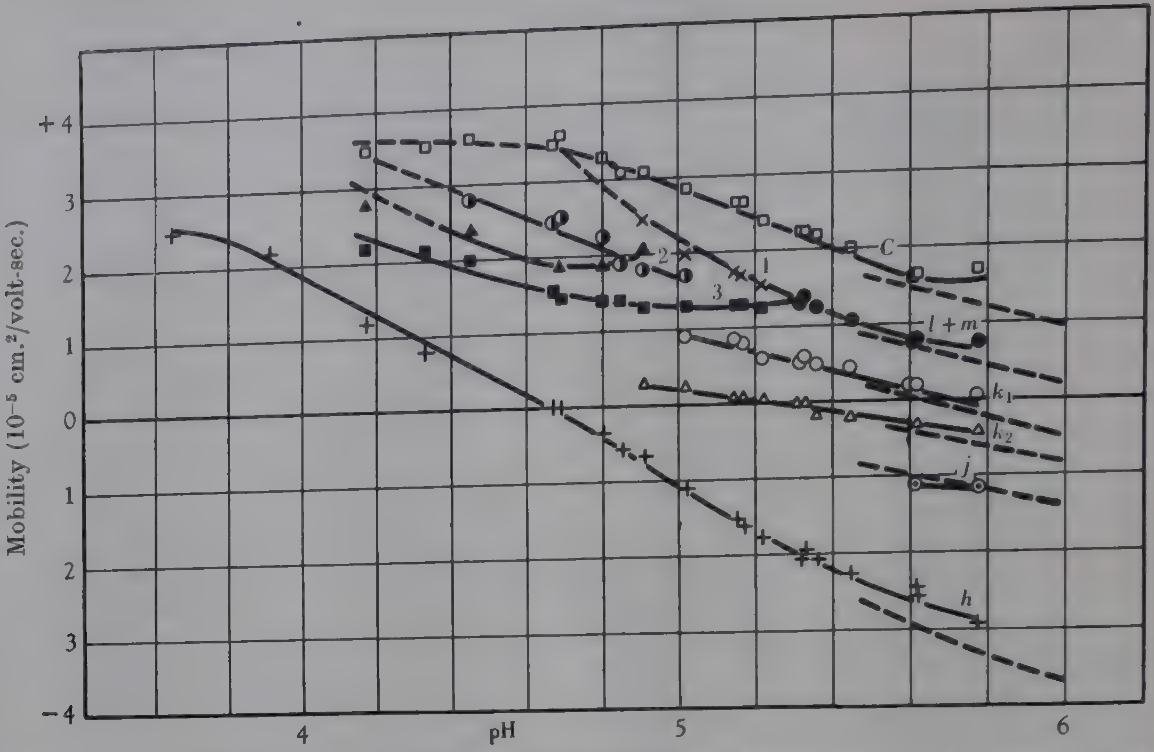

Fig. 3. Descending pH-mobility curves of the electrophoretic components of muscle extracts in acetate buffers of $\mu = 0{\cdot}15$.
□ = *C*, ● = *l* + *m*, × = 1, ◑ = 2, ■ = 3, ▲ = *c*, ○ = k_1, △ = k_2, ⊙ = *j*, + = *h*.

The dashed curves (- - - -) were obtained with phosphate buffers of the same ionic strength (Jacob, 1947; for the pH, see note of Fig. 2 and text, p. 77).

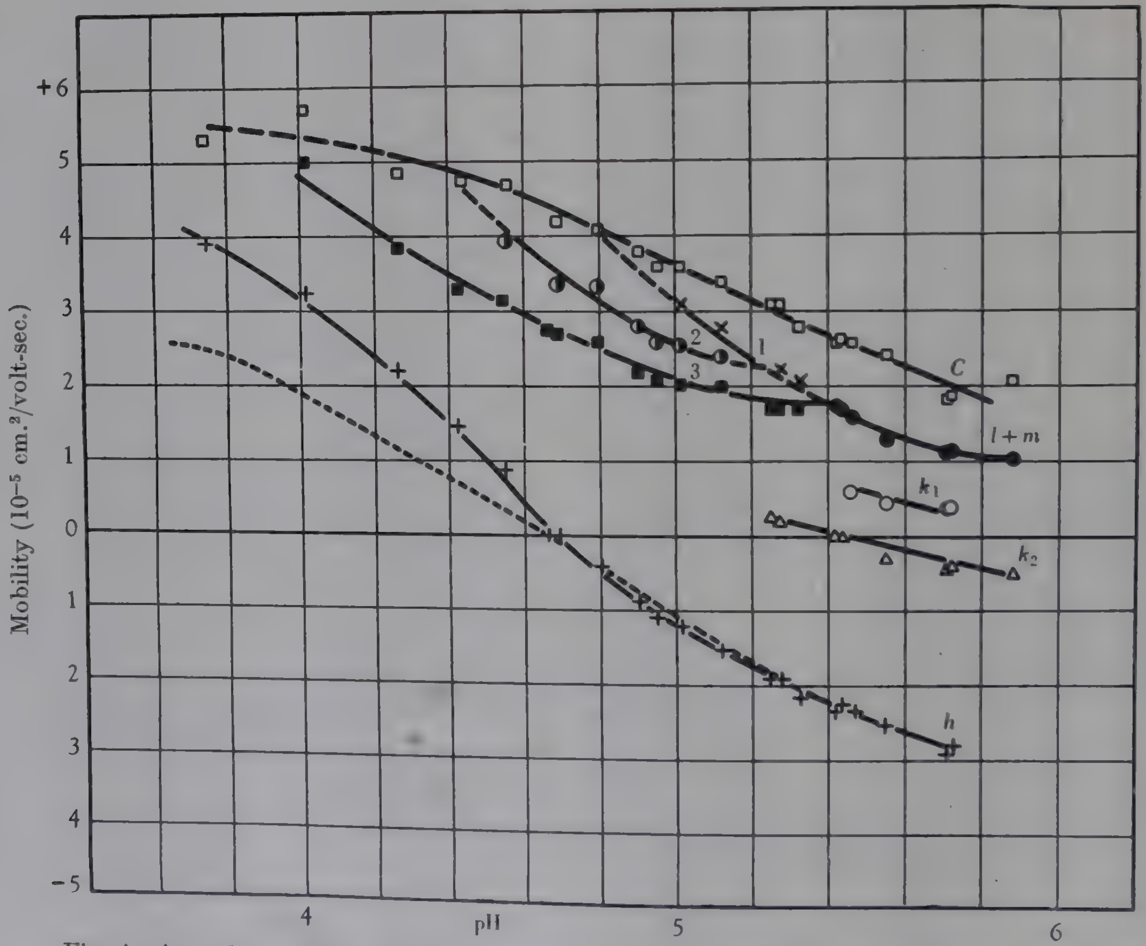

Fig. 4. Ascending pH-mobility curves of the components in acetate buffers of $\mu = 0{\cdot}15$.
□ = *C*, ● = *l* + *m*, × = 1, ◑ = 2, ■ = 3, ○ = k_1, △ = k_2, + = *h*.
The broken (lower) curve (- - -) is the descending curve of *h*.

that aggregation phenomena between proteins are less marked when the protein concentration is low.

Interactions are not likely to be artefacts of the moving boundary method (Perlmann & Kaufman, 1945; Svensson, 1946), for dilution does not result in the disappearance of the asymmetric peak *c*, or a significant reduction of the mobility asymmetries of the various components. It may be concluded that the increase in the area of *C* is due mainly to the formation of a complex. Probably this component interacts with the other components present.

Contrary to facts mentioned by Moyer & Moyer (1940) for rabbit serum proteins, and by Longsworth (1941) for ovalbumin, the pH mobility curves of muscle proteins—in phosphate and acetate buffers—do not diverge noticeably in the common pH zone (Fig. 3). It is worth mentioning that this agreement is found only if the pH values are corrected to 0°, which can be done by using the data of Bates & Acree (1945) for phosphate buffers, and Harned & Ehlers (1932) for acetate buffers.

Recovery of native (or 'reversibly' denatured) proteins from the extracts treated with acetate buffers

The native components *h*, *sp*, *k*, *l*, *m* and *n* of the muscle extracts are soluble in a solution containing 0·032 M-Na_2HPO_4, 0·04 M-NaH_2PO_4 and 0·5 M-NaCl, pH 7·60, $\mu = 0{\cdot}15$ (Jacob, 1947).

If the extracts are first dialyzed against an acetate buffer, centrifuged and then dialyzed a second time against this phosphate solution, a precipitate forms in less than 1 hr. The quantity of protein remaining in solution after removal of the precipitate decreases as the pH of the acetate solution is lowered. The loss is negligible at pH 5·9, but reaches almost 100 % when the pH is 3·64. There is thus a progressive denaturation of the muscle proteins in acetate buffers as the pH falls. This confirms the results of Finn (1932), Weber (1934) and Bate Smith (1937).

The proteins which remain in solution have been examined electrophoretically. Dialysis against phosphate was prolonged (two days, three renewals of the outer liquid), in order to ensure equilibrium between buffer and protein. Its duration does not exceed the limits which were established empirically for the conservation of extracts dialyzed against phosphate buffer alone (Jacob, 1947). In these experiments the identity of the components remaining in solution can be decided from their mobilities, which are very close to those of the components in extracts which were not dialyzed initially against acetate. Very often, they are slightly higher, which may be accidental; there is no evidence of a definite alkaline shift.

The results of the concentration measurements are given in Table 2. If these results are compared with the values obtained for the same extract, after acetate dialysis alone (Table 1), it appears that the loss of concentration is approximately equal to the amount of the complex *C*; and that the amounts of each of the components remaining in solution are close to the amounts of the corresponding peaks of the extract after acetate dialysis alone. This suggests that *C* is the aggregation product of the denatured proteins which precipitate during a second dialysis.

Table 2. *Composition of muscle extracts after two dialyses*

				Relative amounts† of protein					
					Soluble part				
No.*	pH of acetate buffer	pH of phosphate buffer	mg. N/ml. after 2nd dialysis	Ppt.‡	*M* (+*n*)	*l*	k_1	k_2j+sp	*h*
§	—	7·10–7·80	4·0±1·0	0	47·5	24	±7	±12	±5
7	5·33	7·63	1·70	44	18·5	25	5	3	4·5
13	4·85	7·67	1·86	53·5	19	19	2	2	4·5
17	4·34	7·56	0·66	82	4	10·5	1·5		2
19	3·91	7·52	0·32	92	4·0		1·0		3

* This number indicates the extract of Table 1, a part of which was subjected to the second dialysis.

† Relative amounts are calculated relative to the N concentration of the extract after the first dialysis. They can thus be compared directly with the amounts of corresponding experiments in Table 1.

‡ Includes precipitation and experimental losses etc. (see text).

§ Average results of experiments made after a single phosphate dialysis without any detectable preliminary denaturation (Jacob, 1947).

However, the parallelism is not absolute; the loss of concentration represents both the elimination of the precipitate and accidental losses in the course of the second dialysis (osmotic dilution, etc.); it should thus always be greater than the amount of *C*. Yet the opposite is observed in Exps. 13 and 19, and in Exps. 7 and 17 where the loss of concentration is greater than the amount of the complex, this difference being balanced by a fall in the concentration of those peaks which are not yet much involved in the process of progressive aggregation (e.g. *k*, *j*, *sp* and *h* in Exp. 7; *h* in Exp. 7). Presumably the other components remaining in solution are present in a relatively excessive concentration. Consequently, the complex *C* in so far as its real amount is estimated from the descending pattern, is not entirely precipitated. It would thus include, besides the denatured proteins—insoluble in a phosphate buffer—a small proportion of native protein or of denatured proteins soluble in a phosphate buffer.

DISCUSSION

The main object of this study was to characterize the electrophoretic components of extracts from rabbit muscle. In the previous paper (Jacob, 1947), the following isoelectric points were determined: n, $6{\cdot}75+0{\cdot}12$; m, $6{\cdot}20+0{\cdot}12$; l, $6{\cdot}05+0{\cdot}12$; k_1, $5{\cdot}50+0{\cdot}12$ (for the correction $+0{\cdot}12$ see Bates & Acree, 1945). Using acetate buffer, this list is completed with the values: h, 4·65; k_2, 5·25; k_1, 5·70; the isoelectric point of j lies between 4·7 and 5·10.

The isoelectric point of component h is the lowest of all those we have observed. This fraction is not denatured at least down to pH 4·85 (Table 2). This characteristic could not be used to prepare h directly in a purified state; at all pH's lower than 4·90, the elimination of denatured proteins leaves in solution a substantial proportion of proteins other than h. At pH 3·62, the undenatured proteins are very dilute, and electrophoresis of the solution at pH 7·6 has for that reason given no reliable information.

This group of characteristics confirms the identification of peak h with the myoalbumin of Bate Smith. The only divergence relates to the position of the isoelectric point: 3·5 according to Bate Smith (1937), 4·65 on our curve. This divergence may be due either to the use of different buffers at different temperatures, or to the fact that Bate Smith's myoalbumin was modified considerably and adsorbed on quartz particles.

The isoelectric point of h is almost identical with that of serum albumin, but h is not identical with serum albumin, however, as has been shown in a preceding paper (Jacob, 1945).

Similarly, the isoelectric points of j and k_2 differ from those given by Green (1945) for the phosphorylases a and b, which seem to constitute, wholly or partly, these two components respectively (compare the curves and diagrams given by Green with our results, obtained in a phosphate buffer). The divergence is probably due to the fact that this author was compelled to extrapolate more extensively than the present data require.

In group I, the water-soluble part of which corresponds with Weber's myogen (Jacob, 1947) component l is not denatured at least down to pH 5·33. By ultracentrifugation, Gralén (1939) has found that the crystalline myogen A of Baranowski (1939) was stable to a pH of about 5·0; l would correspond mainly to this myogen, which is confirmed by unpublished experiments by Distèche in this laboratory.

The evolution of component n is strange; from pH 5·8 it is wholly included in the denaturation complex. All the evidence seems to indicate that the protein n is a centre of formation for the complex C. It is not impossible that, in a phosphate buffer, n is already a denatured protein (or a protein aggregate) which has undergone an alkaline shift (cf. Neurath, Greenstein, Putman & Erickson, 1944). This hardly seems probable, for in a phosphate buffer, the amount of n does not change much with the pH (Fig. 2). On the other hand, when an extract is denatured in a phosphate buffer by ageing, there is no increase in n, but the turbid matter collects at the level of group II, a group which migrates with a mobility approximately equal to the average mobility of all muscle components (cf. an analogous phenomenon for serum, Davis, Hollaender & Greenstein, 1942). The evolution of n gives rise to many hypotheses which it will be possible to examine only when purified muscle proteins are isolated and studied.

The denaturation of a mixture of muscle proteins in acetate buffers is mainly characterized by the formation of a complex. A similar phenomenon occurs in heated serum (Van der Scheer, Wyckoff & Clarke, 1941; Kleczkowski, 1941), in serum irradiated with ultra-violet rays (Davis *et al.* 1942) and when a mixture of casein and antibody is heated (Krejci, Jennings & Smith, 1942). It is generally admitted that this aggregation is a phenomenon distinct from denaturation proper; it would be made possible by the emergence of new reactive groups in a denatured protein molecule.

The points which characterize the formation of the complex C are: (1) its continuous increase with decrease of pH, (2) the probable existence of interactions between the complex and certain other proteins of the mixture.

If the interpretation given of these interactions is really accurate, complex formation would be an equilibrium reaction, the constant of which would be determined essentially by the pH (or by the concentration of acetic acid). The condition of equilibrium would also be influenced by the concentrations of the proteins involved. This phenomenon can be interpreted either (*a*) as an equilibrium between the original and the denatured forms of a protein (the latter form being the only one liable to enter into the formation of the complex); or (*b*) as a probability of aggregation of molecules which would all be denatured, but to varying degrees, according to the pH (for instance, at a given pH, all molecules are unfolded more than at the immediately higher pH, but less than at the immediately lower pH). This leads us to the hypothesis of Neurath, Cooper & Erickson (1942). The facts presented have, besides, a certain analogy with the experiments on 'reversible' denaturation by urea and guanidine, described by the authors. Indeed, both in Neurath's experiments and in ours the elimination of the denaturing agent brings about the partial precipitation of proteins. This process differentiates the proteins into irreversibly denatured proteins and others which remain soluble, of which in our case we cannot be certain whether they are identical with or slightly different from the original proteins.

We cannot develop any further this idea of equilibrium, and the very numerous problems which it suggests and could solve. The mixture analyzed here is too complex and too poorly defined.

Lastly, the aggregation complex is altered at low pH; this is shown on patterns such as Fig. 1, pH 3·9, which resemble diagrams given by Longsworth (1941) for the acid denaturation of ovalbumin. The interpretation of such asymmetrical diagrams is still unelucidated, but the similarity stresses the analogy of the action of acetic acid, in high concentration, on such widely different proteins as crystalline ovalbumin and a complex of modified muscle proteins.

SUMMARY

1. Protein extracts of rabbit muscles have been submitted to electrophoresis, in buffers containing 0·1 M-Na acetate, 0·05 M-NaCl and various concentrations of acetic acid (total $\mu = 0{\cdot}15$, pH varying between 3·6 and 5·9). As the pH decreases, the patterns diverge more and more from the type of pattern obtained with the same extracts in a phosphate buffer.

2. The analysis of the changes reveals the formation of an aggregation complex and the probability of interactions between this complex and other components of the mixture.

3. If the extracts dialyzed against acetate buffer are then subjected to a second dialysis against a phosphate solution at pH 7·6, a precipitate appears. The latter increases the lower the pH of the acetate buffer, and its amount corresponds approximately to the amount of the aggregation complex formed at that pH.

4. The relation between the electrophoretic components of our extracts and the 'classical' muscle proteins are discussed.

5. The meaning of the aggregation complex (denaturation complex) and its evolution in the course of electrophoresis are interpreted, as far as the great complexity of the observed mixture allows.

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The Conversion of DL-3:5-Diiodo-4-hydroxyphenyllactic Acid into an Analogue of Thyroxine

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In extending the work of Ludwig & von Mutzenbecher (1939) on the iodination of proteins under mildly alkaline conditions, leading to the production of thyroxine within the protein molecule, von Mutzenbecher (1939) illustrated the probable mechanism involved by showing that 3:5-diiodotyrosine, on standing at comparable pH, was converted, in small yield, into thyroxine. The subsequent literature dealing with the conversion of free diiodotyrosine into thyroxine has been cited by Harington & Pitt Rivers (1945; see also Reineke & Turner, 1946), who investigated the conditions of the reaction in some detail and postulated an oxidative mechanism, involving iodine, which they suggest may be of biological significance.

There appears to be no report indicating whether or not derivatives of diiodotyrosine have been found to undergo spontaneous transformation to the corresponding analogues of thyroxine under similar conditions. Of special interest in this connexion is 3:5-diiodo-4-hydroxyphenyllactic acid, which Foster & Gutman (1930) isolated from the urine of rabbits in receipt of large doses of diiodotyrosine. Its identity was ascertained by analysis and by a consideration of its chemical behaviour, but was not confirmed by synthesis.

The present communication is a brief report of the synthesis of DL-3:5-diiodo-4-hydroxyphenyllactic acid, and of the results of preliminary experiments designed to effect its conversion into DL-3:5-diiodo-4-(3′:5′-diiodo-4′-hydroxy)phenoxyphenyllactic acid.

EXPERIMENTAL

Synthesis of DL-3:5-*diiodo-4-hydroxyphenyllactic acid*

The 4-hydroxyphenyllactic acid required for iodination was obtained from the azlactone of 4-acetoxy-α-acetamidocinnamic acid by two-stage hydrolysis to the corresponding pyruvic acid, followed by reduction.

Azlactone of 4-acetoxy-α-acetamidocinnamic acid. Acetylglycine (5·85 g.), 4-hydroxybenzaldehyde (6·1 g.), acetic anhydride (13·4) and fused sodium acetate (3 g.) were refluxed for 1 hr., cooled, and placed overnight at 0°. The mass of yellow-brown crystals was broken up with a rod after the addition of 12 ml. of water. The solid was filtered, washed with ice water and dried *in vacuo* over KOH. Yield (crude): 8·7 g. (85%). The crude material melted at 128°.

4-Acetoxy-α-acetamidocinnamic acid. The crude azlactone (5 g.) was boiled with water (200 ml.) for 10 min. and the hot mixture filtered with gentle suction. The filtrate on cooling deposited yellow crystals which were filtered, washed with water and dried at 110°. Yield: 2·6 g. (48%); m.p. 224°. A specimen recrystallized from water (Norite) gave: N, 5·30%. (Calc. for $C_{13}H_{13}O_5N$, 5·32%.) The hydrolysis of the azlactone with water gave better yields than the acetone-water mixture recommended for the corresponding derivative of benzaldehyde (*Organic Syntheses*, **2**, 519).

4-Hydroxyphenylpyruvic acid. The acetamido acid (2·63 g.) was refluxed for 4 hr. in an all-glass apparatus with 2N-HCl (35 ml.) and placed overnight at 0°. The crystals of the keto acid were filtered and dried *in vacuo.* The crude material (1·0 g. (56%); m.p. 210°; Neubauer (1909), has recorded m.p. 220° for purified material) was reduced soon after preparation as some specimens became pasty on standing.

DL-*4-Hydroxyphenyllactic acid.* The crude keto acid (2 g.) was shaken for 10 min. at room temperature with sodium amalgam (1·25%; 130 g.) and water (25 ml.). The separated and acidified (2N-HCl) solution was extracted thrice with an equal volume of ether. The dried ethereal extract deposited an oil on evaporation, which rapidly solidified to a mass of colourless needles. Yield: 1·3 g. (64%). Recrystallized from ether-light petroleum, the substance melted at 120°, resolidified and then gave a m.p. of 135°. (Erlenmeyer & Lipp (1883) record m.p. 115–122° and 139–140° respectively.) On checking, at a later date, this specimen was found to have m.p. 137–139°, without prior change. Found (after drying at 80°): C, 59·4; H, 5·27. (Calc. for $C_9H_{10}O_4$: C, 59·3; H, 5·49%.)

DL-*3:5-Diiodo-4-hydroxyphenyllactic acid.* The method of iodination used, adapted from that described by Zeynek (1929) for the preparation of 3:5-diiodotyrosine, has been found suitable in this laboratory for the production of a range of diiodophenols. Iodination with iodine monochloride, as utilized by Block & Powell (1943) for the formation of diiodotyrosine, did not yield a satisfactory product.

A solution of 4-hydroxyphenyllactic acid (1·22 g.) in 2N-H_2SO_4 (13·7 ml.) and 10% (w/v) KI (29·4 ml.) was stirred rapidly at 0° with an electric stirrer. Iodic acid (10% (w/v); 15·6 ml.) was then added dropwise from a funnel, followed by glycine (2 g.) in water (30 ml.) to act as a buffer. 2N-NaOH (16 ml.) was then added over 20 min. to adjust the pH to 8 (approx.). Stirring was continued at 0° for 1 hr. and the mixture then allowed to come to room temperature, when it was acidified with 2N-HCl (6 ml.) and extracted thrice with 50 ml. of ether. The ethereal solution was washed with the minimum amount of aqueous $NaHSO_3$ to remove excess iodine and then with water. The oily residue, after removal of the ether from the still moist solution, was extracted successively with 40, 20 and 10 ml. of boiling water. On cooling in ice, 1·25 g. (44%) of colourless needles were deposited. The crystals lost water at 75° and then melted at 164° (Foster & Gutman (1930) report 73–75° and 158–159° respectively for their only partly racemic material). The acid is readily soluble in organic solvents and sparingly soluble in cold water. When boiled in water at pH 7·5 the solution darkened appreciably in colour; after 2 hr. the pH had dropped to 5·5 and it was not found possible to isolate any of the original material. The apparent destruction of the lactic acid under these conditions is in accord with the findings for diiodotyrosine (Harington & Pitt Rivers, 1945; Dickinson & Trikojus, 1947). A specimen was purified for analysis by recrystallization from water and drying at 100° *in vacuo*. Found: C, 24·6; H, 2·08; I, 59·1. (Calc. for $C_9H_8O_4I_2$: C, 24·9; H, 1·84; I, 58·5%.)

Experiments on the transformation of 3:5-*diiodo*-4-*hydroxyphenyllactic acid*

The iodinated lactic acid was incubated at pH (initial) 8·8–9·8 and at temperatures of 37 or 55°, with and without the passage of oxygen. The best yield of transformation product was obtained by the following procedure; a careful comparison with other sets of conditions was not, however, carried out.

The lactic acid (1·0 g.) was dissolved in NaOH (0·1 N; 23 ml.) and 1 N-NaOH added to bring the pH to 8·8. The solution was incubated at 37° for 7 days, a stream of oxygen being provided by passage through a sintered-glass gas distributor. On the 2nd and 4th days *n*-butanol (5 ml.) was added. At the conclusion of the incubation period, sufficient 10 N-NaOH was added to the reaction product to make the aqueous phase normal with respect to this reagent. Further butanol was added to equalize the volumes. The butanolic extract was shaken with an equal volume of 2 N-NaOH and then evaporated to dryness under diminished pressure. The residue was acidified with 2 N-acetic acid and the precipitate purified by dissolving in hot 50% (v/v) acetic acid (1 ml.), filtering from a small residue of amorphous material and then suddenly chilling the hot solution. The yield at this stage was 28 mg. while 350 mg. of pure starting material was recovered. On recrystallization of a portion from dilute acetic acid, an equivalent total recovery of 18 mg. was obtained, the nett yield (about 3%) of this analytically pure material being thus comparable to those obtained by Harington & Pitt Rivers (1945) and Reineke & Turner (1946) in the conversion of diiodotyrosine into thyroxine. Found (after drying at 100°): I, 65·2%. (Calc. for $C_{15}H_{10}O_5I_4$: I, 65·35%.) It crystallized in colourless prisms, m.p. 176–177° (with prior softening), and gave an intense reddish purple colour when treated in *n*-butanol with aqueous alkali and diazotized *N*′-diethylsulphanilamide, a reagent developed in this laboratory for the estimation of thyroxine (Winikoff & Trikojus, 1948). Under appropriate conditions 3:5-diiodo-4-hydroxyphenyllactic acid and 3:5-diiodotyrosine do not give a positive reaction, so that the reagent provides a convenient test in transformations and other studies in this series. The m.p. (173°) recorded for the ketonic acid analogous with thyroxine (Canzanelli, Guild & Harington, 1935) is close to that of our compound, while the iodine value would not differentiate between the two formulae. Although a pure specimen of the keto acid was not available, an examination of the infrared absorption of our compound was undertaken. A solution in morpholine, 5 mg./ml., was examined in the region 5·50–6·50 μ and only one band, at 6·3 μ, characteristic of the carboxylate ion, was found to be present. By contrast, two strong absorption bands, at 5·89 and 6·3 μ, were observed for α-ketoglutaric acid under similar conditions. The first of these is exactly in the correct position for a ketonic carbonyl group. We have, therefore, assumed that the transformation product of 3:5-diiodo-4-hydroxyphenyllactic acid obtained in these experiments is the lactic acid analogue of thyroxine.

SUMMARY

DL-3:5-Diiodo-4-hydroxyphenyllactic acid has been synthesized and its behaviour studied under conditions similar to those which have been found to result in the conversion of diiodotyrosine to thyroxine. A new compound, considered to be the lactic acid analogue of thyroxine, was isolated in a nett yield of about 3%.

While one of us (V. M. T.) was abroad, laboratory facilities, enabling the identification of the transformation product of 3:5-diiodo-4-hydroxyphenyllactic acid to be carried out, were kindly provided by the Director, National Institute for Medical Research, London (Dr C. R. Harington, F.R.S.) and Dr W. Engels of the Research Department, Merck and Co., Inc., Rahway, New Jersey. We are further indebted to Mrs R. V. Pitt Rivers of the former institution for the iodine assay and to Dr N. R. Trenner of Merck and Co. for the infra-red examination and report.

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The Inhibition of Thiol Enzymes by Lachrymators*

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This work was undertaken in order to throw light upon the biochemical mechanism of the lachrymatory effect of certain war gases. Owing to the relationship of one of these, ethyl iodoacetate, to the well known biochemical inhibitor, iodoacetic acid, the study was first directed towards enzymes known to be inhibited by this compound, namely, certain thiol enzymes, or enzymes whose activity appears to depend on the integrity of sulphydryl groups. Iodoacetic acid inhibits muscle glycolysis and yeast fermentation by acting on the triosephosphate dehydrogenase and the yeast alcohol dehydrogenase (Dixon, 1937; Rapkine, 1938; Adler, Gunther & von Euler, 1938). Dickens (1933), and Quastel (1933), showed that iodoacetic acid reacted with thiol groups in cysteine, reduced glutathione and proteins. Since then the list of thiol enzymes has been growing steadily (see Barron & Singer (1945) and Hellermann (1939) for reviews). The following work shows that the lachrymators react with simple thiol groups, that they inhibit almost all the enzymes generally considered to be of a thiol nature, that these enzymes can be protected by free sulphydryl groups, and that most of the enzymes not affected by the lachrymators are believed on other evidence not to contain essential thiol groups. It is suggested that inhibition by the lachrymators may be used as a test for thiol enzymes in general.

METHODS

The lachrymators tested were as follows: ethyl iodoacetate, methyl iodoacetate, bromoacetophenone, chloroacetophenone, bromobenzyl cyanide and chloropicrin. They were dissolved (0·25M) in ethylene glycol monoethyl ether and in all cases the same amount of solvent was added to the control. The experiments were carried out at a temperature of 37° unless otherwise stated.

RESULTS

Reaction with enzymes

Succinic dehydrogenase

The system used for testing this enzyme was prepared from heart muscle, following the method used by Straub (1939) for the preparation of diaphorase, as far as the precipitation at pH 4·6. This precipitate was suspended in a small amount of phosphate buffer pH 7·4, giving a highly active preparation containing all the enzymes necessary for the oxidation of succinic acid by oxygen, namely, succinic dehydrogenase, flavoprotein, cytochrome *c* and cytochrome oxidase. The enzyme was tested in Barcroft manometers, using about 0·1 ml. of enzyme suspension in 1 ml. phosphate buffer pH 7·6 and 1·6 ml. water. KOH papers were used to absorb any CO_2 formed. Sodium succinate (0·4 ml., 20 mg./ml.) was tipped from a Keilin tube at time 0 into the right-hand flask. The lachrymator was tipped in from another Keilin tube either with the substrate or at a given time before it.

Fig. 1 shows various results obtained. The action of the poison is progressive, so that the inhibitory effect depends on both the concentration of the poison and the time of incubation. The rate of inhibition falls off as the reaction between the enzyme and lachrymator proceeds, an equilibrium finally being reached if excess poison is not used.

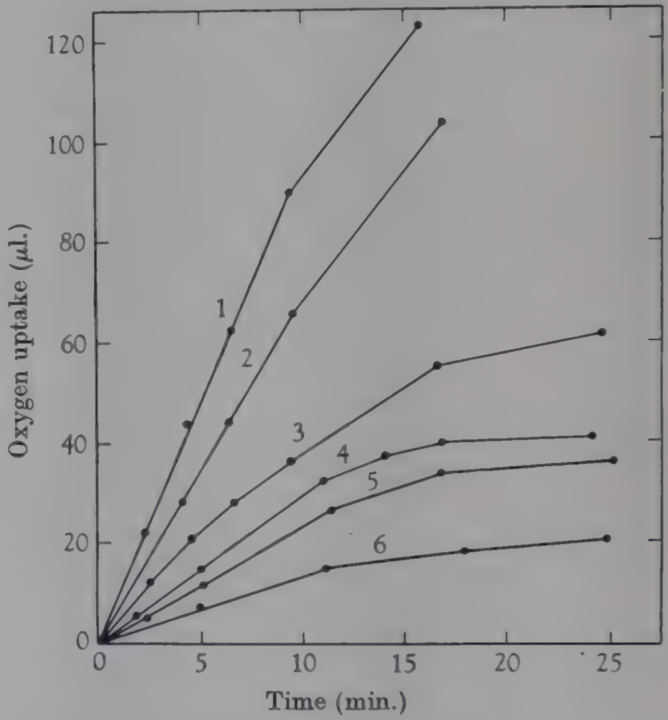

Fig. 1. The effect of lachrymators on the oxygen uptake of the succinic dehydrogenase—cytochrome system. Curve 1, control. Curve 2, M/10,000 ethyl iodoacetate. Curve 3, M/3000 ethyl iodoacetate. Curve 4, M/10,000 bromoacetophenone. Curve 5, M/15,000 chloropicrin. Curve 6, M/10,000 chloropicrin. The lachrymators were added with the substrate at time 0, and the curves show how the inhibition increases with time.

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* This work was carried out in 1940–1 under the direction and supervision of Dr M. Dixon for the Ministry of Supply. It was reported to the Ministry in 1941 (Mackworth, 1941). The author is indebted to the Chief Scientist of the Ministry of Supply for permission to publish this paper.

Ethyl iodoacetate takes longer to reach equilibrium than the other lachrymators. The inhibitory reaction also depends on the presence of any protective substances, especially denatured enzyme, which inevitably occur in varying concentrations in such an enzyme preparation. Therefore any comparative work must be carried out on the same preparation and as far as possible at the same time. Table 1 shows the effects of the different lachrymators on the succinic dehydrogenase—cytochrome system.

Table 1. *Inhibition of succinic dehydrogenase—cytochrome system by lachrymators*

Poison	Conc.	Incubation (min.)	Inhibition (%)
Bromobenzyl cyanide	M/3,000	0	70
		5	85
Ethyl iodoacetate	M/1,500	10	97
	M/3,000	10	90
	M/6,000	10	85
	M/12,000	10	60
Chloropicrin	M/15,000	15	72
	M/50,000	30	68
	M/100,000	15	40
Chloroacetophenone	M/3,000	0	20
		5	42
		20	82

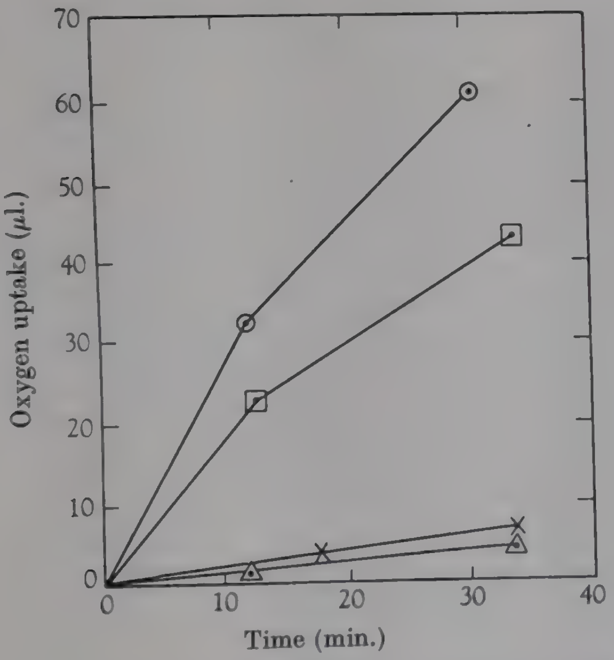

Fig. 2. The effect of lachrymators on succinic dehydrogenase. The cytochrome system has been inhibited by KCN, and methylene blue used in its place. ⊙—⊙ Control; ⊡—⊡ M/10,000 ethyl iodoacetate; ×—× M/10,000 bromoacetophenone; △—△ M/10,000 chloropicrin.

By adding 0·3 ml. 0·01 M-neutralized KCN and 0·3 ml. of 0·5% methylene blue the cytochrome system is inhibited, and the succinic dehydrogenase remains the only active enzyme in the system, methylene blue catalyzing the reaction with oxygen. Fig. 2 shows that the effect of the lachrymators on this system is just as great as before, thus making it clear that the lachrymators attack the dehydrogenase itself. Experiments on the oxidation of *p*-phenylenediamine by the cytochrome oxidase in the preparation show that this enzyme is not affected by M/1500 methyl iodoacetate.

Experiments on protection. It was found that addition of cysteine or reduced glutathione before adding the lachrymator protected the enzyme from the expected inhibition. Considerable excess, about nine times as much thiol as lachrymator, was necessary for complete protection. This may be due partly to the greater affinity of the lachrymator for the enzyme than for the simple thiol, as suggested below, but also it is known that the simple thiols are rapidly oxidized by the enzyme system here used.

Table 2 shows the results, the inhibition being calculated as far as possible from the initial rate over the first 5 min. The enzyme was incubated with the poison, with or without the thiol compound, for 20 min., so that the rate of increase in inhibition during the period of the experiment was usually fairly slow (cf. Fig. 3).

Table 2. *Protection of succinic dehydrogenase by cysteine and reduced glutathione from the effects of the lachrymators*

Poison	Conc.	Conc. of GSH	Initial inhibition (%)
Ethyl iodoacetate	M/5000	0	70
		M/2000	40
Bromoacetophenone	M/9000	0	85
		M/3000	70
		M/2000	58
		M/1000	30
		Conc. of cysteine	
Chloropicrin	M/9000	0	95
		M/1000	20
Bromoacetophenone	M/4500	0	85
		M/1000	25
Bromoacetophenone	M/9000	0	95
		M/3000	75
		M/1000	0

This protection could be used to determine accurately the inhibition produced by the lachrymator after a given time, because by tipping in excess cysteine or glutathione the poisoning could be arrested at any required point. For instance Fig. 3 shows the effect of adding excess glutathione to the succinic system after incubation for 20 min. with M/3000 ethyl iodoacetate. In the flask in which glutathione (GSH) has not been added the inhibition continues to increase, whilst in the flask in which GSH is added with the substrate, the inhibition is arrested, and remains at that level at which it was in both experiments when the substrate was added. These results show that although GSH protects the enzyme, there is no reversal, and in fact no evidence of reversal was ever obtained in working with lachrymators on succinic dehydrogenase.

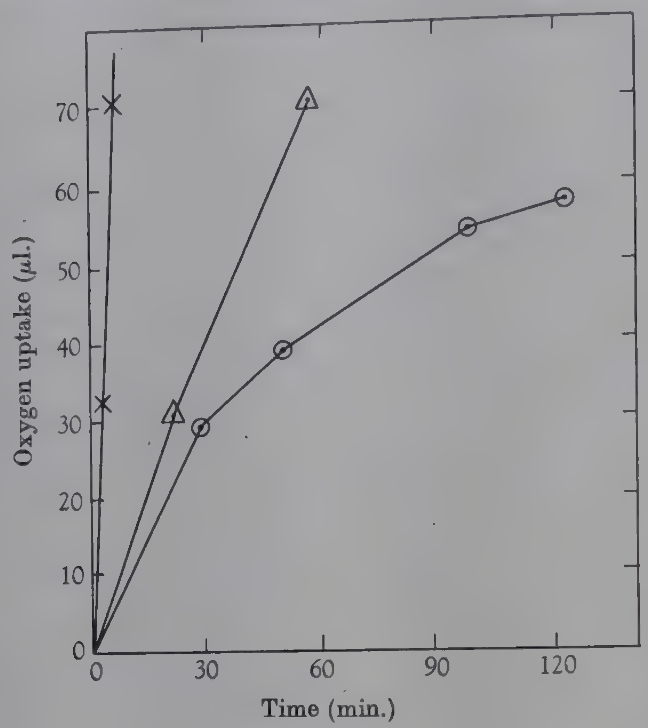

Fig. 3. Protection of succinic dehydrogenase from the effects of ethyl iodoacetate by reduced glutathione. ×—× Control; △—△ after 20 min. incubation with M/3000 ethyl iodoacetate, excess GSH is tipped in with the substrate at time 0; ⊙—⊙ after incubation with ethyl iodoacetate the substrate alone is tipped in at time 0.

Since it thus appears that the poisoning is arrested immediately the thiol compound is added, it is possible to obtain figures for the relation between the time of action of the poison and the activity of the enzyme. Table 3 shows the results of adding cysteine (M/1500) at varying times after the addition of bromoacetophenone to succinic dehydrogenase. It appears that the rate of poisoning is not uniform, but falls off until no further poisoning can be obtained over a considerable period of time. This is the result that might be expected if the rate of inhibition depended on the concentrations of both the enzyme and the poison, and if both were destroyed during the reaction. However, destruction of the poison by something other than the enzyme would produce similar results, and it is clear from the slightly varying levels of inhibition produced by the poison on different preparations containing the same amounts of active enzyme that such destruction does occur. Only experiments with pure enzyme could clarify this point.

Table 3. *Effect of terminating bromoacetophenone poisoning of succinic dehydrogenase with cysteine*

Conc. of bromoacetophenone	Time of incubation before cysteine added (min.)	Activity remaining (%)
M/62,000: Exp. I	2	82
	5	45
	10	22
Exp. II	4	50
	8	45
	16	29
M/37,000	2	35
	5	17
	7	17

The most interesting protection is that by boiled enzyme. It was found that 0·2 ml. of boiled inactive enzyme would completely protect an equal amount of the active enzyme from poisoning by M/9000 bromoacetophenone, which without the boiled enzyme produced an inhibition of 95%.

Choline dehydrogenase

The enzyme was prepared from rat liver (Mann, Woodward & Quastel, 1938) by grinding in a mortar with phosphate buffer pH 7·4. The suspension was then homogenized in the Booth-Green bacterial mill for 15–20 min., centrifuged, and the fluid dialyzed against running tap-water for 1–2 hr. This preparation keeps for about 2 days at 0°. The oxidation of choline using 1 ml. of enzyme was followed either by oxygen uptake in Barcroft manometers or by methylene blue reduction in evacuated Thunberg tubes. The preparation contained all the factors necessary for the oxidation of choline by oxygen.

Table 4 shows the effects of the lachrymators after 20 min. incubation. As with succinic dehydrogenase, the inhibition was progressive, and the results show the initial inhibition when the substrate was first added, measured over the first 5–10 min.

Table 4. *The effects of lachrymators on choline dehydrogenase*

Conc. of poison		Inhibition (%)
A. In Barcroft manometers:		
Chloroacetophenone	M/2500	65
	M/1500	95
Bromobenzyl cyanide	M/2500	80
Bromoacetophenone	M/2500	90
	M/4500	90*
Ethyl iodoacetate	M/1500	—
(No incubation)		20
(20 min. incubation)		80
B. In Thunberg tubes:		
Bromobenzyl cyanide	M/6000	50
	M/3000	60
	M/2000	75

* Obtained with a specially purified preparation.

Table 5 shows the protection afforded by cysteine against M/1500 chloroacetophenone, after 20 min. incubation with the poison, as measured in Barcroft manometers. In this experiment it was found that 0·2 ml. of boiled enzyme was sufficient to give complete protection.

Table 5. *Protection of choline dehydrogenase by cysteine against* M/1500 *chloroacetophenone*

Conc. of cysteine	Inhibition (%)
0	95
M/500	82
M/250	45

Yeast alcohol dehydrogenase

The enzyme was prepared by the first step of the method of Negelein & Wulff (1937), which gives a very active preparation. Juice prepared from dried yeast was heated, cooled and a half volume of acetone was added to the centrifugal fluid. The precipitate was discarded and a further half volume of acetone was added. This precipitate, containing the enzyme, was dried over H_2SO_4 and dissolved in water for use. It was tested in Thunberg tubes with methylene blue. In order to complete the system, cozymase and diaphorase were added and semicarbazide to remove the acetaldehyde formed. The enzyme was incubated with the lachrymators for 15 min. before the ethanol and dye were tipped from the stopper.

Table 6 shows that the lachrymators have a considerably greater effect on the enzyme than has iodoacetate.

Papain

British Drug Houses papain was used, and activated by adding 30 mg. cysteine in solution at pH 4·8 to 5 ml. of 0·5% papain solution. The substrate used was fresh milk plus 0·2 vol. of 0·2 M-acetate buffer, the mixture having a pH of 4·6.

The reaction with ethyl iodoacetate is complicated by the necessity of activating papain with cysteine which would react with the iodoacetate; while if the papain and iodoacetate were incubated together before addition of cysteine, it seemed likely, from what is already known about papain, that the thiol group in the enzyme might be masked in a disulphide linkage. This proved to be the case, as the following results show. Activated papain (1 mg.) clotted the milk in 2 min., whereas 10 mg. natural papain were necessary for this clotting time. When incubated with 0·01 M-ethyl iodoacetate before activation and then treated with M/60 cysteine, 20 mg. of papain were necessary for the same speed of clotting, whereas if first activated with 0·02 M-cysteine, incubated with 0·02 M-ethyl iodoacetate and then treated with 0·04 M-cysteine, no activity remained.

This showed that the papain could be regarded as less susceptible to the ethyl iodoacetate before activation, as was to be expected if in the inactive form the thiol group were combined in an S—S linkage.

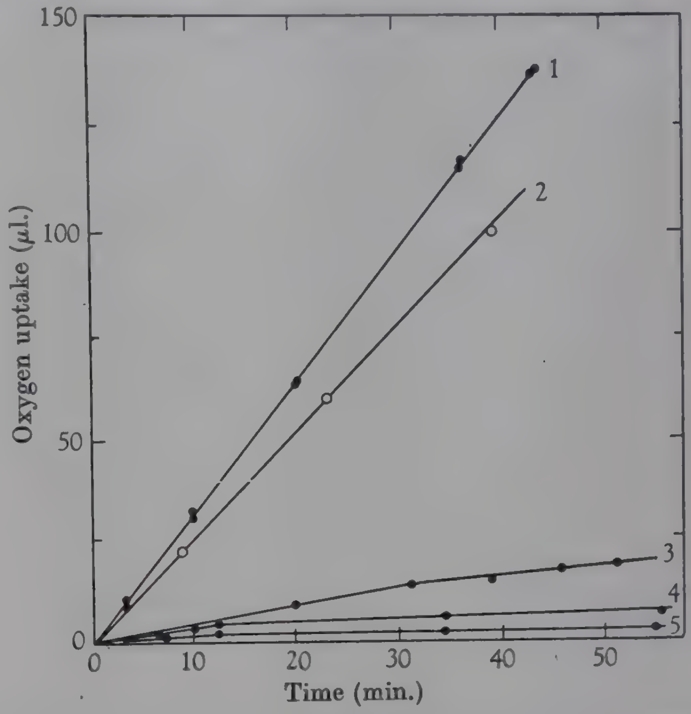

Fig. 4. The effect of the lachrymators on pyruvic oxidase of *Esch. coli*. Incubation 20 min. Curve 1, control and with M/1600 sodium iodoacetate. Curve 2, M/6000 ethyl iodoacetate. Curve 3, M/1600 methyl iodoacetate. Curve 4, M/3000 chloropicrin. Curve 5, M/3000 bromoacetophenone.

Pyruvic oxidase

The enzyme was prepared from *Esch. coli* by the method of Still (1941) and tested in Barcroft manometers as described by him. Fig. 4 shows the results.

Table 6. *The effect of lachrymators on yeast alcohol dehydrogenase*

	Time of reduction of methylene blue		Inhibition (%)
	min.	sec.	
Control	1	45	—
+ Na iodoacetate (M/3000)	2	35	60
+ ethyl iodoacetate (M/3000)	>60	—	100
+ ethyl iodoacetate (M/6000)	8	—	85
+ bromoacetophenone (M/3000)	>60	—	100
+ chloropicrin (M/3000)	>60	—	100
+ bromobenzyl cyanide (M/3000)	>60	—	100
+ chloroacetophenone (M/3000)	8	—	85

Triosephosphate dehydrogenase

A 5% suspension of muscle acetone powder, prepared as described by Green, Needham & Dewan (1937), was centrifuged and the soluble portion used as the source of the enzyme and of the other enzymes necessary for the testing reaction. The substrate was added in the form of hexosediphosphate solution, which is rapidly converted into triosephosphate by the zymohexase present in the extract. This latter enzyme is not inhibited by the lachrymators. The reaction was carried out in Barcroft manometers in bicarbonate solution equilibrated with a gas mixture containing 5% CO_2 in N_2. Coenzyme I was added as hydrogen carrier, and pyruvate to accept the hydrogen removed from the triosephosphate. The phosphoglyceric acid formed was measured by the CO_2 evolution.

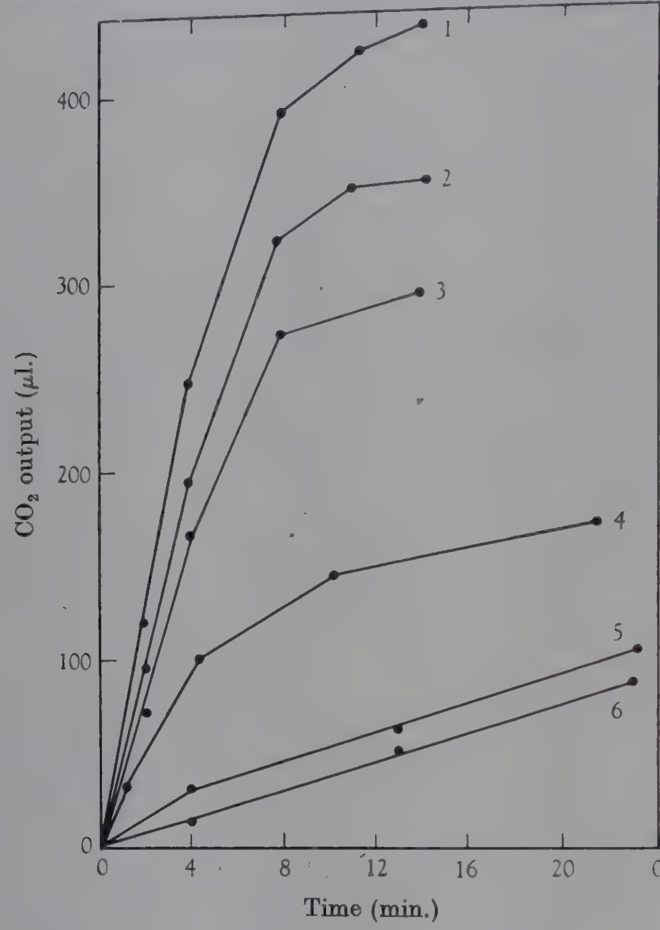

Fig. 5.

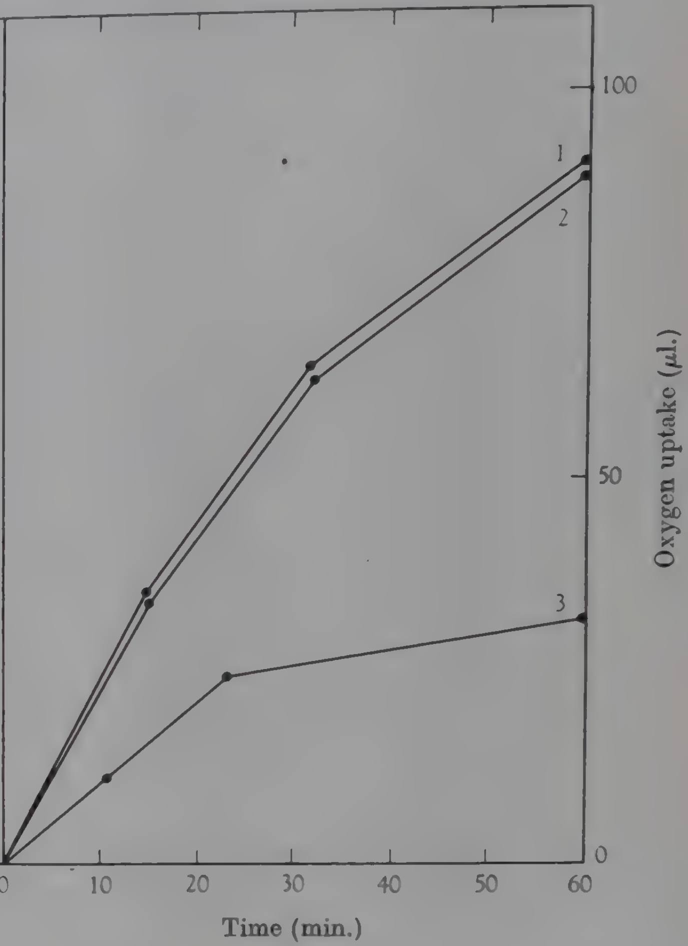

Fig. 6.

Fig. 5. The effect of lachrymators and sodium iodoacetate on triosephosphate dehydrogenase. Incubation 20 min. Curve 1, control for curves 2 and 3. Curve 2, M/3000 chloropicrin. Curve 3, M/3000 bromacetophenone. Curve 4, control for curves 5 and 6. Curve 5, M/3000 sodium iodoacetate. Curve 6, M/3000 ethyl iodoacetate.

Fig. 6. The effect of methyl iodoacetate and sodium iodoacetate on xanthine oxidase. Incubation 15 min. Curve 1, control. Curve 2, M/660 sodium iodoacetate. Curve 3, M/660 methyl iodoacetate.

Table 7. *The effect of lachrymators on triose phosphate dehydrogenase*

Poison	Conc.	Inhibition (%)
Na iodoacetate	M/3000	90
	M/5000	60
Methyl iodoacetate	M/3000	90
	M/5000	50
Ethyl iodoacetate	M/3000	90
Chloropicrin	M/3000	20
Bromobenzyl cyanide	M/2000	45
Bromoacetophenone	M/2000	50
	M/3000	30
Chloroacetophenone	M/1000	30

Table 8. *The effect of the lachrymators on xanthine oxidase, tested in Thunberg tubes*

Poison		Time of reduction of methylene blue		Inhibition (%)
		min.	sec.	
None		—	50	—
Bromobenzyl cyanide	(M/1200)	10	—	93
Bromoacetophenone	(M/625)	8	—	85
	(M/2400)	4	30	80
Chloropicrin	(M/1200)	1	45	66
	(M/500)	3	—	75

This enzyme differs from all the other thiol enzymes in being quite as sensitive to the iodoacetate ion as to the lachrymators. Fig. 5 shows the effects of M/3000 iodoacetate and some of the lachrymators. The two experiments recorded here have different controls owing to the varying activity of the muscle powder. Because the control reaction is not linear, the degree of inhibition is not readily determined, but Table 7 shows the initial inhibitions obtained after 20 min. incubation with the lachrymators.

Xanthine oxidase

The enzyme was used in the form of a whey preparation as described by Dixon & Kodama (1926) and the oxidation of hypoxanthine by oxygen followed in Barcroft manometers. Portions of 1·6 ml. of a 10% solution of the enzyme were added to both flasks and also 0·05 ml. of an active preparation of catalase, to destroy any hydrogen peroxide formed. Hypoxanthine (1 mg.) was added from a Keilin tube at time 0. The enzyme was incubated with methyl iodoacetate for 15 min. before adding the substrate.

Fig. 6 shows the effect of M/660 methyl iodoacetate as compared with the same concentration of sodium iodoacetate.

The other lachrymators were tested by the Thunberg tube method, using methylene blue. Table 8 shows the results.

Cholinesterase

The enzyme was prepared in a purified form from horse serum by the method of Stedman & Stedman (1935). The hydrolysis of acetylcholine was followed in Barcroft manometers, by measuring the amount of CO_2 set free from bicarbonate-Ringer solution by the free acid formed. After 15 min. incubation with the lachrymators 3 mg. of acetylcholine in neutralized solution were tipped in.

Table 9 shows the effects of the lachrymators on this enzyme after 30 min. incubation. It is clear that it is much less sensitive than any of the previous enzymes. A crude extract of rabbit's brain, prepared by grinding the brain with sand and pressing through muslin, was also tested, but the brain enzyme was found to be unaffected by M/1250 bromoacetophenone.

Table 9. *The effect of lachrymators on cholinesterase from horse serum*

Poison	Conc.	Inhibition (%)
Bromobenzyl cyanide	M/900	25
	M/365	70
Bromoacetophenone	M/1250	Nil
	M/365	60
Chloropicrin	M/365	Nil
Ethyl iodoacetate	M/600	Nil

Urease

Urease tablets (0·1 g.) were extracted with 10 ml. phosphate buffer pH 7·4 and the insoluble part filtered off. The activity was measured by the method of Sumner (1926), slightly modified. 1 ml. of a solution containing 5·4 g. K_2HPO_4, 4·25 g. KH_2PO_4 and 0·15 g. urea/100 ml. was added to 1 ml. of the enzyme solution, and after standing at room temperature for the required time, the ammonia content of the samples was estimated with Nessler solution.

It was found that after 15 min. incubation with M/400 ethyl iodoacetate 50% inhibition was produced, while M/600 bromoacetophenone gave 80% inhibition. The enzyme was therefore not as sensitive as might be expected from its known thiol nature, but this might have been due to impurities in the solution.

Carboxylase

The CO_2 liberated by dried yeast extract acting on pyruvate was measured in Barcroft manometers. An inhibition of about 20% was obtained by incubating the yeast with M/1500 ethyl iodoacetate for 15 min. before adding the substrate.

Glyoxalase

The enzyme was prepared from horse liver and tested by the method of Lohmann (1932). The methylglyoxal was placed in the flasks of Barcroft manometers with the enzyme, the glutathione being tipped in from Keilin tubes.

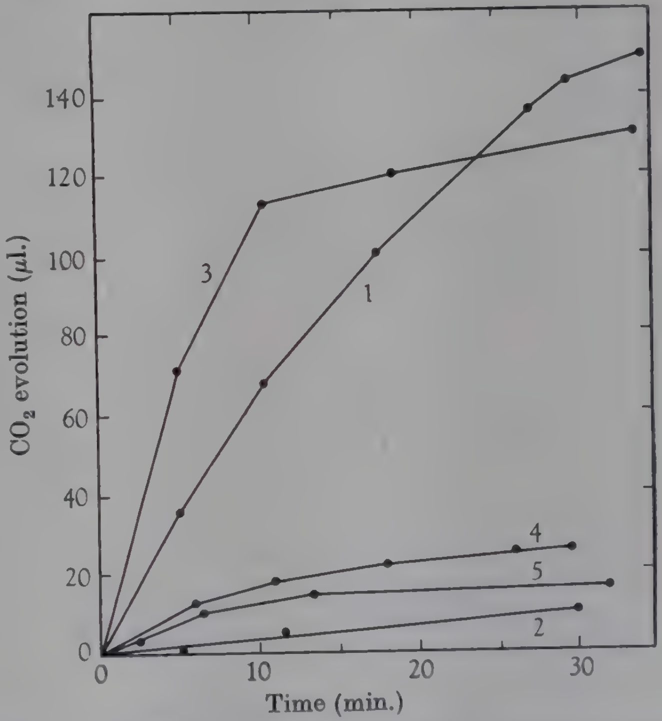

Fig. 7. The effect of the lachrymators on glyoxalase and its coenzyme, reduced glutathione. Curve 1, enzyme and 50 µg. GSH. Curve 2, 50 µg. GSH incubated with 300 µg. bromoacetophenone before addition to the enzyme. Curve 3, M/2300 bromoacetophenone incubated with the enzyme, before 400 µg. GSH were added. Curve 4, 50 µg. GSH incubated with 500 µg. ethyl iodoacetate before being added to the enzyme. Curve 5, 50 µg. GSH incubated with 260 µg. chloropicrin.

Fig. 7 shows the results of treating the enzyme or the coenzyme, reduced glutathione, with lachrymators. In curve 1, 50 µg. of GSH were added to the enzyme from a Keilin tube at time 0. Curve 2 shows the effect of incubating the GSH in the Keilin tubes with 300 µg. of bromoacetophenone before tipping. The concentration of lachrymator was *c*. M/70 in the Keilin tube and, after tipping, M/2300 in the flask. In curve 3, M/2300 bromoacetophenone was incubated with the enzyme and then 400 µg. of GSH were tipped in. While the incubation of the lachrymator with the GSH has almost completely destroyed the activity, treatment of the enzyme appears not to inhibit. This is due to the fact that the rate depends partly on the concentration of GSH. Curves 4 and 5 show the destruction of GSH by incubating 50 µg. GSH with 500 µg. ethyl iodoacetate and 260 µg. chloropicrin respectively. It is clear that all three lachrymators destroy GSH.

Metabolism

Muscle respiration

Pigeon-breast muscle or rat-leg muscle was minced in a Latapie mincer with the grinding die removed, and suitable amounts (usually about 200 mg.) were weighed directly into the manometer flasks. The solution contained 1 mg. of succinate to stabilize the respiration. Any non-cyclic oxidation of succinate would be finished before readings were taken. The method was that of Stare & Baumann (1936). The lachrymators were tipped in during the experiment so that the rates of O_2 uptake of each portion of tissue both before and after the addition of the poison were known. Portions of chopped rat-leg muscle were similarly treated.

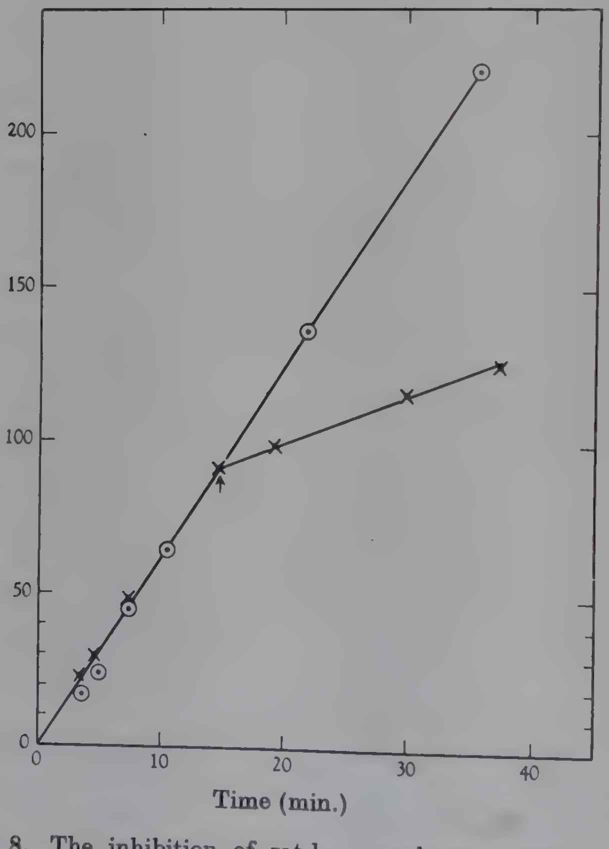

Fig. 8. The inhibition of rat-leg muscle respiration by bromoacetophenone. ⊙—⊙ Control; ×—× as control at the beginning, M/2000 bromoacetophenone being added at the arrow.

Fig. 8 shows the effects on the respiration of rat-leg muscle of adding 0·0005 M-bromoacetophenone at the point indicated by the arrow. While the control continued at the rate already established, the muscle treated with the lachrymator immediately ceased to respire. Similar results were obtained with pigeon-breast muscle, of which the respiration was almost completely inhibited by M/1500 ethyl iodoacetate.

Yeast fermentation

The fermentation of glucose by baker's yeast was followed in Barcroft manometers by measuring the CO_2 produced in the absence of O_2, the flasks being filled with N_2 freed from O_2 by passing over heated copper. The lachrymators were tipped in from Keilin tubes so that accurate measure of the time of incubation could be obtained.

All the lachrymators, except chloropicrin, inhibit glucose fermentation by yeast, the inhibition being progressive and irreversible. The inhibition produced by 0·0002 M-ethyl iodoacetate, bromoacetophenone or bromobenzyl cyanide becomes almost complete in about 20 min. Chloroacetophenone produces about 60 % inhibition in 40 min. The inhibition produced by 0·0002 M-chloropicrin reached 30 % in 5 min., but did not increase during the next 20 min. The reason for this will be explained in the next section.

Yeast respiration in glucose

The oxygen uptake of baker's yeast was measured in Barcroft manometers, KOH papers being used to absorb the CO_2 formed. 17 mg. of glucose were used as substrate although the respiration would normally continue without added glucose. In order to measure carbohydrate respiration solely the yeast was starved for 24 hr., when the residual respiration without added glucose was very small.

Table 10 shows the inhibition produced after 30 min. incubation, the initial rate of respiration at this time being determined. The inhibition of respiration was progressive, except with chloropicrin. When starved yeast was treated with M/750 methyl iodoacetate, it was found that the poison had no effect on the small oxygen uptake (30 µl. in 90 min.) which occurred without the addition of a substrate, but when 17 mg. of glucose were added, the great increase in respiration (200 µl. in 15 min.) which took place in the control was completely absent in the poisoned yeast, whose respiration remained at the same level as the control yeast without added glucose.

Table 10. *The effect of the lachrymators on yeast (glucose) respiration*

(Conc. poison, M/6000; incubation 30 min.)

Poison	Inhibition (%)
Chloropicrin	40
Chloroacetophenone	70
Ethyl iodoacetate	82
Bromobenzyl cyanide	88
Bromoacetophenone	95

Ethyl iodoacetate, incubation 10 min.

Conc.	Inhibition (%)
M/1,500	85
M/3,000	80
M/6,000	75
M/12,000	65
M/24,000	55
M/48,000	35

It will be noticed from this table that chloropicrin, in marked contrast to its effect on other enzymes, has only a very slight effect on yeast. The reason for this appears to be that yeast can destroy chloropicrin; when treated with 0·001 M-chloropicrin, the initial inhibition of 60% is no longer shown after washing on the centrifuge, and the poisoned yeast becomes as active as the control. Similar treatment of ethyl iodoacetate-poisoned yeast produces no reactivation. Moreover, if M/750 chloropicrin is incubated with yeast for 2 hr. and then the solution is added to the succinic dehydrogenase system, no inhibition at all is obtained, showing that the chloropicrin must have been completely destroyed. Chloropicrin incubated without yeast—and bromoacetophenone incubated with yeast—both gave complete inhibition of the dehydrogenase system. This is the only case found in dealing with lachrymators in which there is evidence of reversal of the inhibition, and it seems likely that it is due to a special enzyme in the yeast, which can remove the chloropicrin from its combination with the inhibited enzyme.

Table 11 shows the enzymes which have been found to be unaffected by the lachrymators in the concentrations shown.

Table 11. *Enzymes not affected by the lachrymators*

Enzyme	Poison	Conc. tested
Cytochrome oxidase	Methyl iodoacetate	M/1500
Carbonic anhydrase	Bromoacetophenone	M/1500
Diaphorase	Methyl iodoacetate	M/300
D-Amino-acid oxidase	Methyl iodoacetate	M/750
Zymohexase	Methyl iodoacetate	M/1500
Glucose dehydrogenase	Ethyl iodoacetate	M/1500
Lactic dehydrogenase (muscle)	Methyl iodoacetate	M/1500
Malic dehydrogenase	Methyl iodoacetate	M/300

DISCUSSION

The foregoing experiments suggest that the action of the lachrymators on the body may be due to inhibition of thiol enzymes, many of which, such as succinic dehydrogenase and pyruvic oxidase, are known to play an important part in the general metabolism of the body. From an analogy with the iodoacetate ion (Dickens, 1933), it is probable that the lachrymators combine irreversibly with the enzyme, their toxicity being dependent on the halide group. They are far more toxic to almost every enzyme than iodoacetic acid, and can be regarded as reliable thiol reagents, when applied in low concentrations. Triosephosphate dehydrogenase shows a marked exception to this generalization, for it is as sensitive to ionic iodoacetate as to its esters, while the other lachrymators have a smaller effect. The only example observed of reversibility is in the inhibition of yeast by chloropicrin.

On the assumption that the lachrymators react only with the thiol group it is suggested that xanthine oxidase, an enzyme not hitherto known to belong to the thiol class, should be included in this class. It has long been known that this enzyme is very sensitive to oxidation, especially by hydrogen peroxide (Dixon & Kodama, 1926), and recently Mann & Quastel (1946) have shown that it is inhibited by oxygen at 1 atm. They suggest that this might be due to the formation of hydrogen peroxide, but in the experiments shown in this paper the peroxide was destroyed by catalase, so that it is more probable that the oxidase is a true thiol enzyme. This is the only flavoprotein which appears to be sensitive to the lachrymators.

There are many enzymes which are completely insensitive to the lachrymators, such as cytochrome oxidase and lactic dehydrogenase, and all workers are agreed that these enzymes are insensitive to all thiol reagents. There are a few enzymes which are only slightly sensitive, such as cholinesterase. Nachmansohn & Lederer (1939) suggested that this was a thiol enzyme, but they found it no more sensitive to thiol reagents than it is to the lachrymators. Finally there is the considerable class of thiol enzymes, which are very sensitive to lachrymators. Dickens (1946) has shown how these enzymes are sensitive to high oxygen pressures, and among them he includes choline oxidase, shown here to be a thiol enzyme. He finds that D-amino-acid oxidase is not sensitive to high oxygen pressure, nor is it sensitive to the lachrymators, although it is sensitive to arsenicals (Barron & Singer, 1945).

The lachrymators appear to react more readily with proteins containing thiol groups than with simple thiols, such as cysteine. While about nine molecules cysteine/molecule lachrymator are required for complete protection, the denatured

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enzyme preparation will give protection in concentrations which contain no appreciable thiol groups demonstrable by the nitroprusside test. The combination with the denatured enzyme preparation must have been much faster than with the active enzyme, since if there had been any appreciable competition some of the latter must have been destroyed, and this protective action might be expected on the theory that denaturation increases the number of reactive thiol groups; denatured egg white, however, although it is known to contain numerous thiol groups, had no protective power.

The experiments with papain support the theory that this enzyme contains a thiol group which in the inactive form is probably present as S—S. When the partially inactive form was treated with ethyl iodoacetate and activated after this treatment, the inhibition was not complete. In contrast to this, when the papain was first activated with cysteine and then poisoned, complete inhibition was obtained, although excess cysteine was added after the poison.

The action of yeast on chloropicrin is of considerable interest. It appears that the yeast is capable of destroying the chloropicrin, and that washing can produce complete reactivation of the yeast. As this is the only case in which reversal has been obtained, experiments might be carried out on other enzymes, to see whether yeast could reactivate them when they have been poisoned with chloropicrin. It suggests that reversal might be a question of finding a reactant with a sufficiently powerful affinity for the lachrymator.

SUMMARY

1. An account is given of the effects of various lachrymators on enzyme systems. It is shown that they inhibit all the enzymes which are generally accepted as thiol enzymes, the inhibition being progressive and irreversible in all cases except that of chloropicrin on yeast.

2. The enzymes can be protected against the inhibition by reduced glutathione, cysteine, or denatured enzyme. A considerable excess of the simple thiols is required for complete protection.

3. It is shown that the lachrymators inhibit the respiration of muscle. They also inhibit equally the fermentation and respiration of glucose by yeast.

4. It is suggested that the lachrymators act like iodoacetate in that they combine irreversibly with thiol groups in the enzyme, but they are much more powerful than iodoacetate.

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The Inhibition of Serum Cholinesterase by Alkyl Fluorophosphonates*

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During the war, a team of workers under Drs Saunders and McCombie prepared a series of fluorophosphonate esters and related compounds (McCombie & Saunders, 1946). Some of them were found to be highly toxic to animals by inhalation and very rapid in action, and to have a powerful myotic action when present in sub-lethal concentrations. In man, exposure to amounts of vapour insufficient to give any sensory effect produced a pupillary contraction and spasm of accommodation lasting several days. It was suggested that the parasympatheticomimetic activity of the fluorophosphonates, like that of eserine, might be due to an inhibition of cholinesterase. The results reported here have made it clear that these esters do in fact act like eserine in poisoning the enzyme, and that this reaction *in vitro* can be used as a test for the pharmacological potency of the esters.

EXPERIMENTAL

Materials and methods

Enzyme. Cholinesterase was prepared from horse serum by the method of Stedman & Stedman (1935), carrying the preparation as far as the dialysis against chloroform water. The method of estimation was essentially that of Ammon (1933). The enzyme solution was placed in the right-hand flask of a Barcroft manometer, in a total volume of 3 ml. 0·2% $NaHCO_3$; the gas phase was 5% CO_2 in N_2. The reaction, carried out at 20°, was started by tipping in a solution containing 2 mg. acetylcholine chloride. The CO_2 output was usually linear until about 100 μl. had been produced. The activity remained remarkably constant for many weeks when the stock enzyme was stored at 0° in presence of a little chloroform. For the inhibition experiments, sufficient enzyme was taken to produce 100–120 μl. CO_2 in 10 min. at 20°.

Poisons. The fluorophosphonates and related compounds were obtained from Dr B. C. Saunders; eserine sulphate was a commercial product (British Drug Houses Ltd.). Since fluorophosphonates slowly hydrolyze in water, stock solutions of these and of eserine were prepared in ethylene glycol monoethyl ether, and diluted with the same solvent so that an addition of 0·01–0·03 ml. to the 3 ml. of enzyme-buffer solution in the manometer vessel gave the required final concentration of poison. The enzyme, buffer and poison were incubated in the manometer vessel for the required time (15 min. in the standard test mentioned below) at 20° before tipping in the substrate from dangling tubes. For the experiments with a short duration of incubation, the poison solution was tipped from a dangling tube and the acetylcholine from a side tube.

The activity of the treated enzyme was compared against that of a control incubated with the solvent alone. Since it was found that the enzyme was unaffected by the solvent, or by incubation at 20° without substrate for a period of some hours, it was not necessary to repeat the control with every set of estimations.

RESULTS

Progress curve of inhibition. In preliminary experiments with di*iso*propyl fluorophosphonate, it was found that concentrations above 10^{-7} M completely inhibited cholinesterase almost instantaneously. With lower concentrations, the inhibition produced varied with the time of incubation. Fig. 1 shows the inhibition of cholinesterase by di*iso*propyl fluorophosphonate, and by a comparable amount of eserine, after varying times. The action of eserine reaches a maximum within 5 min., while the inhibition by fluorophosphonate is initially less rapid, but is progressive and ultimately more complete. The latter effect suggests an irreversible inactivation of the enzyme rather than an equilibrium.

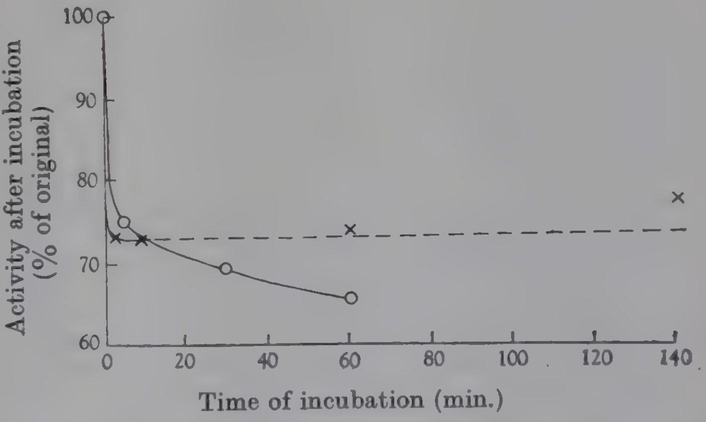

Fig. 1. Progress curve of inhibition of horse serum cholinesterase by eserine and by di*iso*propyl fluorophosphonate, in absence of substrate, at pH 7·4 and 20°. ×—×, 5×10^{-8} M-eserine; ○—○ *c.* 3×10^{-10} M-di*iso*propyl fluorophosphonate.

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* This work formed part of the research programme carried out for the Ministry of Supply by an Extra-Mural Research Team under the direction of Dr M. Dixon, and was reported to the Ministry of Supply (Mackworth, 1942; Webb, 1944).

Reversibility. It was shown by Matthes (1930) that the effect of eserine on cholinesterase could be reversed completely by prolonged dialysis against water. These results have been confirmed, but on the other hand it proved impossible to obtain any reversal of the poisoning by the fluorophosphonate esters (Table 1). The enzyme solution (5 ml.) was treated with the inhibitor for 15 min. at 38°; 1 ml. was used at once for activity estimation, and the remainder dialyzed against running tap water for 24 hr. in the case of the eserine experiment, 36 hr. in the others. A control was treated in the same manner, in the absence of inhibitor, and corrections were applied for the volume change on dialysis. It is clear that the combination between the fluorophosphonate esters and the enzyme is of a much firmer nature than that between eserine and the enzyme.

Table 1. *Effect of prolonged dialysis against water on activity of cholinesterase poisoned with eserine and fluorophosphonates*

(Enzyme incubated with poison for 15 min. at 38° before dialysis.)

Inhibitor	Conc. (M)	Inhibition (%) Before dialysis	Inhibition (%) After dialysis for 24–36 hr.
Eserine	10^{-6}	100	35
Diethyl fluorophosphonate	4×10^{-9}	70	76
Diethyl fluorophosphonate	10^{-8}	90	85
Di*iso*propyl fluorophosphonate	10^{-9}	50	50

Effect of substrate concentration. In the following experiments the cholinesterase activities were measured by a continuous titration method. The digest of acetylcholine and horse serum cholinesterase (total vol. 10 ml.), containing bromothymol blue and 0·0002 M-phosphate, was titrated with 0·01 N-NaOH to maintain the pH at 7·4. The titrations, which were carried out in a water bath at 20°, were linear over a period of 10–15 min. The velocity was expressed as ml. 0·01 N-NaOH/5 min.; under the conditions used, it was proportional to the enzyme concentration. When an inhibitor was added, this was equilibrated with the enzyme etc., for 5 min. at 20° before adding the substrate contained in a volume of 1 ml.

Under the conditions used the enzyme was markedly less sensitive to fluorophosphonate than in the manometric experiments, due probably to the higher enzyme concentration and the shorter incubation period, so that only the initial rapid inhibition took place. With an acetylcholine concentration of 0·0045 M, 50 % inhibition was produced by approximately 2×10^{-7} M-eserine or $3{\cdot}5\times10^{-8}$ M-di*iso*propyl fluorophosphonate. When the substrate concentration was varied over the range 0·0004–0·06 M, the percentage inhibition by the fluorophosphonate (compared with a standard having a similar substrate concentration but no inhibitor) remained more or less constant. On the other hand, the inhibition due to eserine decreased when the acetylcholine concentration was raised. The difference in the behaviour of the two inhibitors is clearly shown when the results are examined by the method of Lineweaver & Burk (1934), which has already been applied to serum cholinesterase and eserine by Eadie (1942). The relationship between enzyme reaction velocity v and substrate concentration S is given by

$$\frac{1}{v}=\frac{1}{V}+\frac{K_p}{V}\frac{1}{S},$$

where V is the limiting velocity and K_p is the effective Michaelis constant. In the presence of a 'competitive' reversible inhibitor, V is unchanged but K_p is increased, i.e. the apparent affinity of the enzyme for its substrate is lowered. If there is no competition between inhibitor and substrate for the enzyme centres, $K_p=K_M$ but V is lowered.

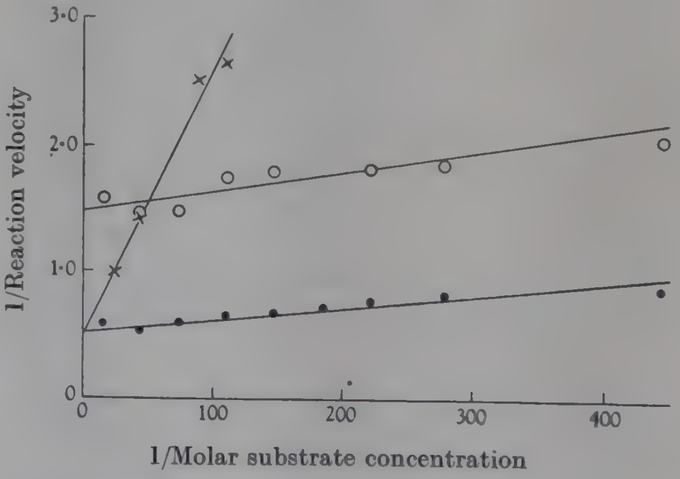


Fig. 2. Effect of substrate concentration on inhibition of horse serum cholinesterase. Enzyme activity estimated by titration with 0·01 N-NaOH at pH 7·4 and 20°. ●—●, control, no inhibitor; ×—×, 2×10^{-7} M-eserine; ○—○, 5×10^{-8} M-di*iso*propyl fluorophosphonate.

Fig. 2 shows the results obtained with 2×10^{-7} M-eserine and 5×10^{-8} M-di*iso*propyl fluorophosphonate. Eserine behaves like a typical competitive inhibitor, with *V* unchanged, but the fluorophosphonate gives no indication of competition with the substrate.

True and pseudo-cholinesterase. The crude serum preparation used contained both the 'true' and 'pseudo' cholinesterases of Mendel & Rudney (1943). The effect of di*iso*propyl fluorophosphonate on these components was examined separately by means of the specific substrates described by Mendel, Mundell & Rudney (1943), using the titration method described above. Fluorophosphonate (5×10^{-8} M) gave an inhibition of 57 % of the activity towards 0·0045 M-acetylcholine, 30 % of the activity towards 0·0005 M-acetyl-β-methylcholine, and 40 % of that towards 0·005 M-benzoylcholine, after incubating the enzyme with the poison for 5 min. Thus there is no appreciable difference in sensitivity of the true and pseudo-cholinesterases of horse serum to fluorophosphonates.

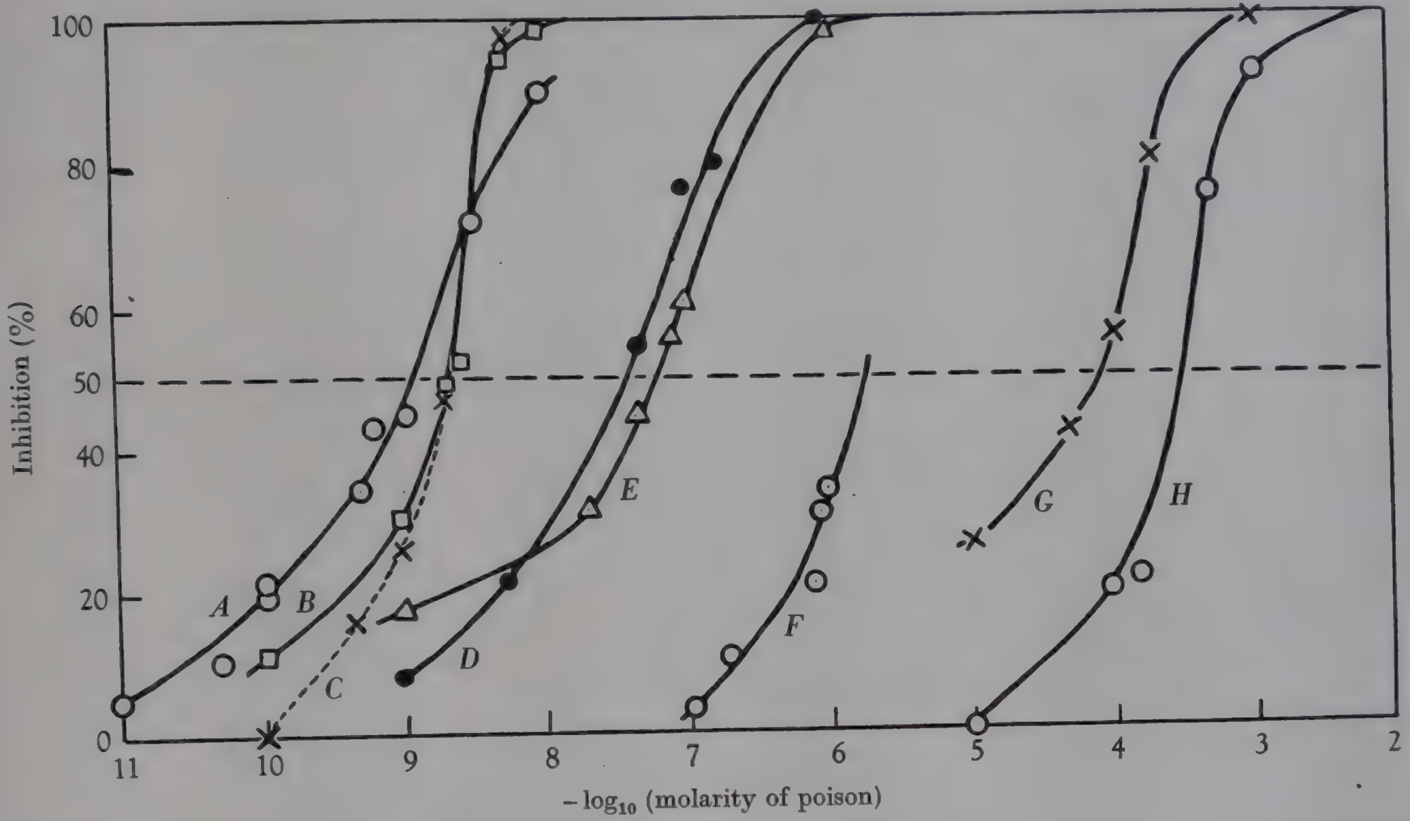


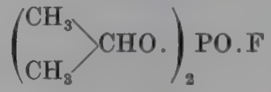
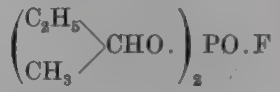
Fig. 3. Inhibition of horse serum cholinesterase by various compounds. Incubated for 15 min. at 20° before addition of 2 mg. acetylcholine chloride. A, di*iso*propyl fluorophosphonate; B, di*sec.*-butyl fluorophosphonate; C, di*iso*amyl fluorophosphonate; D, eserine; E, diphenyl fluorophosphonate; F, dithioethyl fluorophosphonate; G, dimethylaminophosphoryl fluoride; H, diethyl methylaminophosphate.

Assessment of inhibitory power. In order to compare the inhibitory power of the different compounds, conditions for a standard manometric test were defined. A fixed amount of enzyme was incubated at 20° with varying concentrations of the inhibitor for 15 min. before tipping in the acetylcholine. The CO_2 production in the first 10 min. was used for estimating the activity, and by comparison with a control, the percentage inhibition. Curves showing the percentage inhibition produced under the standard conditions plotted against the logarithm of the inhibitor concentration for eserine and a number of alkyl fluorophosphonates are shown in Fig. 3. They are in general sigmoid in shape, but are not all similar; in particular, those for *iso*propyl and *sec.*-butyl fluorophosphonate are considerably flatter than the others, so that in the range of inhibitions below 50 % these esters are relatively more effective than would be indicated by the pI_{50} values (see below). With different preparations of cholinesterase the absolute positions of the curves tended to vary, but they occupied the same position relative to one another. The graph for eserine agrees with that given by Ellis, Krayer & Plachte (1943). In an extended series of studies, the inhibition-concentration curves for new esters were always compared against that for di*iso*propyl fluorophosphonate, which was redetermined at the same time.

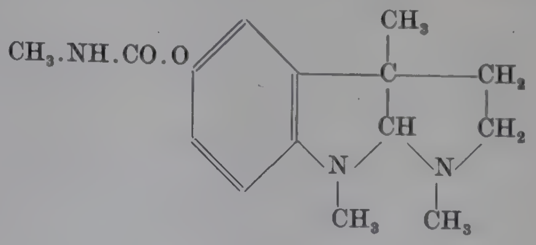
To obtain a numerical value for inhibitory power, the concentration of poison producing 50 % inhibition in the standard test was read off from the inhibition-log (concentration) curve. This value is conveniently expressed as the pI_{50}, which is the negative logarithm of the concentration producing 50 % inhibition (Mazur & Bodansky, 1944). Values for the poisons examined are given in Table 2. Di*iso*propyl fluorophosphonate was the most active of these compounds, being thirty times more potent than eserine. The other branched-chain esters, namely *sec.*-butyl and *iso*amyl, although less active than the *iso*propyl derivative, are more active than the straight-chain *n*-propyl, ethyl and methyl esters. This suggests a special affinity of the enzyme for methyl groups, which are also present in the substrate and in eserine. Replacement of oxygen in diethyl fluorophosphonate by sulphur reduces the activity to 1/400 of the original. A slight activity is shown by methylaminophosphonate ethyl ester; unesterified fluorophosphonate is virtually inactive.

Table 2. *Inhibitory power of eserine, fluorophosphonates and related compounds on horse serum cholinesterase*

(Standard test conditions; 15 min. incubation at 20° in bicarbonate buffer, pH 7·4, in absence of substrate.)

Inhibitor	Formula	Molarity producing 50 % inhibition	pI_{50} ($-\log_{10}$M)
Di*iso*propyl fluorophosphonate	 $\left(\begin{matrix}CH_3\\CH_3\end{matrix}>CHO.\right)_2PO.F$	$1·3\times10^{-9}$	8·9
Di*sec.*-butyl fluorophosphonate	 $\left(\begin{matrix}C_2H_5\\CH_3\end{matrix}>CHO.\right)_2PO.F$	$2·0\times10^{-9}$	8·7
Di*iso*amyl fluorophosphonate	$\left(\begin{matrix}C_2H_5\\CH_3\end{matrix}>CH.CHO.\right)_2PO.F$	$2·0\times10^{-9}$	8·7
Di-*n*-propyl fluorophosphonate	$(CH_3.CH_2.CH_2O.)_2PO.F$	$5·5\times10^{-9}$	8·25
Diethyl fluorophosphonate	$(C_2H_5O.)_2PO.F$	$8·0\times10^{-9}$	8·1
Eserine	*	$4·0\times10^{-8}$	7·4
Diphenyl fluorophosphonate	$(C_6H_5O.)_2PO.F$	$6·3\times10^{-8}$	7·2
Dimethyl fluorophosphonate	$(CH_3O.)_2PO.F$	$1·0\times10^{-7}$	7·0
Dithioethyl fluorophosphonate	$(C_2H_5S.)_2PO.F$	$2·0\times10^{-6}$	5·7
Dimethylaminophosphoryl fluoride	$(CH_3)_2N.PO(OH).F$	$8·0\times10^{-5}$	4·1
Diethyl methylaminophosphonate	$(C_2H_5O.)_2PO.NHCH_3$	$3·0\times10^{-4}$	3·5
Trimethyl phosphate	$(CH_3)_3PO_4$	$1·0\times10^{-3}$	3·0
Vitamin B_1	—	$1·7\times10^{-3}$	2·8
Ammonium fluorophosphonate	$(NH_4O.)_2PO.F$	$1·0\times10^{-2}$	2·0
Sodium fluoride	NaF	$1·0\times10^{-2}$	2·0

* Eserine has the following structure (Polonovski & Polonovski, 1925):



serum cholinesterase and serum lipase activities *in vivo*. Similarly, Mazur & Bodansky (1946) have observed a correlation between serum cholinesterase and poisoning by fluorophosphonates, although Hawkins & Gunter (1946) have shown that complete inhibition of the serum (pseudo) cholinesterase *in vivo* is not necessarily accompanied by symptoms of acetylcholine accumulation. The values obtained in this work for inhibitory power towards serum cholinesterase fall into the same series as those from the physiological assay on the rabbit eye, although there is not a strict quantitative agreement. Possibly better agreement would have been obtained if the compounds had been assessed on a tissue true cholinesterase.

DISCUSSION

The extremely high affinity for cholinesterase of the fluorophosphonates, especially those with branched-chain alkyl groups, suggests that the toxic and myotic power of these compounds is due to inhibition of cholinesterase *in vivo*. Bloch (1943) showed that the parasympatheticomimetic symptoms of poisoning by cresyl phosphate, which is also an inhibitor of cholinesterase *in vitro*, were paralleled by a fall of

The inhibition of cholinesterase by eserine is readily understood by comparing the structure of the alkaloid with that of acetylcholine. Eadie (1942) has shown that eserine is a competitive inhibitor of cholinesterase, and that an equilibrium is reached within a few minutes of mixing, in which two molecules of eserine combine with one molecule of enzyme. The action of other inhibitors which possess a strongly dissociated quaternary ammonium group, such as methylene blue, miotine (Massart & Dufait, 1941), phenylpyrazolones (Zeller, 1942), and vitamin B_1, which is a quaternary thiazolinium salt (Glick & Antopol, 1939), is probably similar. The action of the alkyl fluorophosphonates, on the other hand, is probably of a different nature; it is not reversible and there is no evidence of substrate competition or of an equilibrium, the destruction being progressive for at least an hour. Nevertheless the sigmoid curves obtained when inhibition is plotted against the logarithm of inhibitor concentration simulate equilibrium curves, and the more flattened, asymmetric curves obtained for the most potent esters resemble those which Straus & Goldstein (1943) have shown are to be expected for enzyme-inhibitor equilibrium when the enzyme concentration is high relative to the enzyme-inhibitor dissociation constant (so that the degree of inhibition is dependent to some extent on the concentration of the enzyme, as well as that of the inhibitor). Probably similar curves are to be expected if the degree of inhibition is determined by the velocity of a reaction between enzyme and inhibitor, dependent on their respective concentrations. It is possible that the initial rapid inhibition is an equilibrium, but that this is followed by an irreversible destruction of the enzyme; this would be consistent with the two-phase nature of the progress curve of inhibition. In any case, the effective irreversibility of the destruction would explain the prolonged action of the fluorophosphonates *in vivo*, in contrast to the transitory effect of eserine.

Easson & Stedman (1936) and Straus & Goldstein (1943) give figures of 1490 and 450 molecules of acetylcholine/sec./enzyme centre respectively for the absolute activity of horse serum cholinesterase at 30°, based on the inhibition by eserine at varying concentrations. (These figures depend on the assumption that there is an equilibrium between one enzyme centre and one molecule of eserine. They presumably need recalculation in the light of the work of Eadie mentioned above, but are probably of the right order of magnitude.) In the manometric experiment with an amount of serum cholinesterase which produced 100 μl. CO_2/10 min. at 20°, complete inhibition was produced by about 10^{-8} M-di*iso*propyl fluorophosphonate. Calculation shows that under these conditions complete inactivation was produced in 15 min. by the presence of 1–3 molecules of fluorophosphonate/enzyme active centre, which is particularly remarkable in view of the low purity of the enzyme preparation used. Clearly the fluorophosphonate has a very specific affinity for the active centres attacked.

SUMMARY

1. The alkyl fluorophosphonates examined were found to be highly potent inhibitors of horse serum cholinesterase. The most active ester, di*iso*propyl fluorophosphonate, was 30 times as active as eserine.

2. The inhibition of cholinesterase by alkyl fluorophosphonates, unlike the action of eserine, is progressive with time. It is not affected by the substrate concentration and cannot be reversed by prolonged dialysis.

3. The most potent esters were those with short branched-chain alkyl groups. The order of inhibitory power of the various esters was roughly the same as that for toxicity and for myotic power.

4. The true and pseudo-cholinesterase components of horse serum were equally sensitive to di*iso*propyl fluorophosphonate.

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The Action of Alkyl Fluorophosphonates on Esterases and other Enzymes

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Work carried out during the war showed that alkyl fluorophosphonates, especially those with short branched-chain alkyl groups, have a very powerful inhibitory action on serum cholinesterase *in vitro* (Mackworth & Webb, 1948), and it was at first supposed that this action might be specific for cholinesterase. To test this hypothesis, the action of a potent alkyl fluorophosphonate was studied on a number of enzymes *in vitro*, and the results are reported in this paper. They show that esterases other than serum cholinesterase are sensitive to fluorophosphonate.

EXPERIMENTAL AND RESULTS

Tissue cholinesterase. The cholinesterase activity of a rabbit brain preparation, obtained by grinding the brain in bicarbonate Ringer solution and straining through muslin, was estimated in Barcroft manometers in the presence of 8 mg. acetylcholine. Under the conditions of treatment reported in the previous paper for serum cholinesterase, the brain enzyme was 50% inhibited by $6{\cdot}7 \times 10^{-8}$ M-ethyl fluorophosphonate, or by 5×10^{-7} M-eserine. Thus the inhibitory activity of diethyl fluorophosphonate against rabbit brain cholinesterase ($pI_{50} = 7{\cdot}2$) is about one eighth of its activity against horse serum cholinesterase ($pI_{50} = 8{\cdot}1$) but the relative activities of the two inhibitors on each enzyme remains the same.

Liver esterase. Simple esterase was estimated, with ethyl butyrate as substrate, by the continuous titration method of Harrer & King (1941). The titration mixture (10 ml.) contained M/450 ethyl butyrate, M/5000 phosphate and 0·003% bromothymol blue, and was maintained at pH 7·4 by titration with 0·01 N-NaOH at 20°. The enzyme had been partially purified from an aqueous extract of acetone-dried ox liver by fractional precipitation with ammonium sulphate and fractional adsorption on calcium phosphate gel. It had no detectable action on acetylcholine (cholinesterase activity/simple esterase activity < 1/1500).

Table 1 shows the inhibition of the enzyme by fluorophosphonate, eserine, and fluoride. To prevent reversal of the inhibition on dilution, the enzyme was incubated with the poison in a volume of 2 ml. for 15 min. at 20°, and then the reaction was started by adding 8 ml. of substrate, phosphate, etc., containing the same concentration of poison as the enzyme solution. It will be seen that liver esterase is strongly inhibited by fluorophosphonate but is relatively insensitive to eserine and fluoride.

Table 1. *Inhibition of liver esterase*

(Ethyl butyrate as substrate. Enzyme incubated with poison for 15 min. at 20° before estimation.)

Inhibitor	Conc. (M)	Inhibition (%)
Di*iso*propyl fluorophosphonate	10^{-6}	91
	5×10^{-7}	77
	3×10^{-7}	32
	10^{-7}	<10
Eserine	10^{-2}	65
	10^{-3}	25
NaF	10^{-2}	>85
	2×10^{-3}	73
	10^{-3}	48
	10^{-4}	7

Milk lipase. Human milk contains a powerful lipase, which can easily be estimated by a continuous titration method (Hottinger & Bloch, 1943). The most convenient substrate was a 1·5% emulsion of tributyrin in 1% sodium glycocholate (British Drug Houses Ltd,), neutralized to pH 7·5. A sample of human milk (4 weeks *post partum*) was skimmed by centrifuging and used directly as the enzyme; 0·06 ml. was sufficient for an activity estimation with tributyrin. The enzyme, together with 0·5 ml. 0·02 M-phosphate and 0·3 ml. 0·1% indicator (bromothymol blue or cresol red) was diluted to 8 ml., and the reaction started by addition of 2 ml. of the tributyrin emulsion. The mixture, contained in a boiling tube, was shaken gently and continuously in a water bath at 20°, and 0·01 N-NaOH was run in from a micro-burette to maintain the pH constant. The titration was completely linear over a period of 10–15 min. for a total addition of 1–2 ml. NaOH, the velocity being directly proportional to the amount of enzyme taken. Control titrations, (*a*) without enzyme and (*b*) without substrate, were zero. The optimal pH was found to be 8·0 (± 0·2), and the experiments with poisons were carried out at pH 8 with cresol red as indicator, after a preliminary 15 min. incubation of enzyme and poison at pH 7·4. A few experiments were carried out with other substrates, and the activities obtained are shown in Table 2.

Table 2. *Hydrolytic activity of skimmed human milk on various substrates*

(At pH 8, 20°, in presence of 0·2% glycocholate. 1 unit = the amount of enzyme which produces free acid equivalent to 1 ml. 0·01 N-NaOH in 5 min.)

Substrate	Activity (units/ml. milk)
Tributyrin	17·5
Olive oil	0·25
Ethyl butyrate	0·28
Acetylcholine	⩽0·004

The tributyrinase activity was strongly inhibited by fluorophosphonate, considerably less so by eserine, and was insensitive to fluoride. The inhibition by eserine increased continuously, even after addition of substrate, to a maximum at 2 hr. (Table 3). Similar results were obtained with olive oil as substrate.

Kidney phosphatase. The enzyme was prepared from rabbit kidney by the method of Delory & King (1944), and was estimated by the method of Bodansky (1933), using sodium β-glycerophosphate as substrate. The acid phosphatase activity was only moderately sensitive to fluorophosphonate, the alkaline phosphatase still less so (Table 4). According to Belfanti, Contardi & Ercoli (1935) acid phosphatase is sensitive to fluoride, while alkaline phosphatase is not.

Yeast pyrophosphatase. The purified yeast pyrophosphatase of Bailey & Webb (1944) was 48% inhibited by 10^{-3} M-di*iso*propyl fluorophosphonate, and unaffected by eserine. However, it was far more sensitive to free fluoride (50% inhibition by 2×10^{-5} M-NaF), and the result obtained with fluorophosphonate might be due to about 2% of free fluoride present as impurity in the preparation.

Enzymes insensitive to fluorophosphonate. Diethyl and dimethyl fluorophosphonates were found to be without effect on the following enzymes, when tested in concentrations up to 0·002 M: choline dehydrogenase, cytochrome oxidase, catalase, peroxidase, succinic dehydrogenase, lactic dehydrogenase (of muscle), alcohol dehydrogenase (of liver), yeast hexokinase, carboxylase, amylase, diaphorase, carbonic anhydrase, and the glycolytic system of muscle.

Table 3. *Inhibition of human milk lipase*

(At pH 8, 20°; in 0·2 % glycocholate, 0·001 M-phosphate. Preliminary incubation at pH 7·4.)

Substrate	Inhibitor	Concentration (M)	Time of incubation without substrate (min.)	Inhibition (%)
Tributyrin	Di*iso*propyl fluorophosphonate	10^{-4}	15	100
		10^{-5}	15	66
		5×10^{-6}	15	50
		2×10^{-6}	15	32
		10^{-6}	15	16
Tributyrin	Eserine	10^{-3}	15	35–60
		10^{-3}	120	>97
		3×10^{-4}	120	86
		10^{-4}	120	50
Tributyrin	NaF	10^{-1}	15	40
		10^{-2}	15	0
Olive oil	Di*iso*propyl fluorophosphonate	10^{-5}	15	77
Olive oil	Eserine	10^{-3}	15	60–95

Table 4. *Inhibition of kidney phosphatases*

(38°, 0·02 M-glycerophosphate. Acid phosphatase estimated in 0·1 M-acetate, pH 5·5. Alkaline phosphatase estimated in 0·08 M-veronal-acetate buffer pH 9, containing 0·005 M-$MgCl_2$. Enzyme and poison incubated for 15 min. before addition of substrate.)

Inhibitor	Concentration (M)	pH	Inhibition (%)
Di*iso*propyl fluorophosphonate	10^{-3}	5·5	60
	3×10^{-4}	5·5	5
	10^{-4}	5·5	0
Eserine	10^{-3}	5·5	18
Di*iso*propyl fluorophosphonate	10^{-3}	9·0	12
Eserine	10^{-3}	9·0	7

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Table 5. *Sensitivity of various esterases to fluorophosphonate ester, eserine and fluoride*

($pI_{50} = -\log_{10}$ (molarity producing 50% inhibition).)

Enzyme	pI_{50}		
	Di*iso*propyl fluorophosphonate	Eserine	NaF
Serum cholinesterase	8·9*	7·4*	2·0*
	7·45	6·7	—
Liver esterase	6·4	*c.* 2·5	*c.* 3
Human milk lipase	5·3	*c.* 3 (15 min. incubation)	<1
	—	*c.* 4 (2 hr. incubation)	—
Kidney acid phosphatase	*c.* 3	<3	(+)
Kidney alkaline phosphatase	$\leqslant 3$	$\leqslant 3$	(−)
Yeast pyrophosphatase	3 (?)	0	4·7

* These values were calculated from manometric results; all the others were obtained by a titration method.

DISCUSSION

It is clear that the fluorophosphonates are not, as was at first supposed, specific inhibitors of cholinesterase. They poison a range of esterases, some of which have no activity at all towards acetylcholine. In this they resemble tri-*o*-cresyl phosphate and tri-*o*-chlorophenyl phosphate, which have been shown to inhibit serum and tissue cholinesterase, serum lipase, and human milk lipase, but not pancreatic lipase (Bloch, 1943; Hottinger & Bloch, 1943). Bloch has drawn attention to the fact that the enzymes which are inhibited by cresyl phosphate are also sensitive to eserine. However, Table 5 shows that there is no quantitative relationship between the sensitivity of enzymes to fluorophosphonate and to eserine, or between the sensitivity to fluorophosphonate and to free fluoride.

Mackworth & Webb (1948) in the preceding paper gave reasons for believing that the action of fluorophosphonates on serum cholinesterase is of quite a different nature from the action of eserine on the enzyme. The distinction is emphasized by the differing specificity of inhibition reported above; in particular, by the powerful action of fluorophosphonate on simple esterase and milk lipase, which are comparatively insensitive to eserine.

SUMMARY

1. Brain cholinesterase was inhibited by an alkyl fluorophosphonate, but not as strongly as serum cholinesterase.

2. Liver esterase, human milk lipase and kidney phosphatase were also sensitive to di*iso*propyl fluorophosphonate, though not to the same extent as cholinesterase. These enzymes were also inhibited by eserine, but there was no correlation between the sensitivity towards fluorophosphonate and towards eserine or sodium fluoride. Liver esterase in particular is highly sensitive to fluorophosphonate but relatively insensitive to eserine. The specificity of inhibition by fluorophosphonates is similar to that reported for tri-*o*-cresyl phosphate and tri-*o*-chlorophenyl phosphate (Bloch, 1943).

3. Alkyl fluorophosphonates had no effect on a number of other enzymes tested, with the possible exception of yeast pyrophosphatase.

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The Synthesis of Some Dipeptides Related to Gramicidin S

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This paper describes the synthesis of five dipeptides required for study of the structure of gramicidin S (Consden, Gordon, Martin & Synge, 1947). For all the syntheses carbobenzoxy derivatives were employed according to Bergmann's general procedure; coupling was effected in each case through the acid chloride. Fair yields were obtained at all stages, and no special difficulties were encountered. This appears to be the first time that peptides of valine or of ornithine have been synthesized by the carbobenzoxy procedure. For synthesizing α-(L-valyl)-L-ornithine it was necessary to block the δ-amino group of ornithine with the carbobenzoxy radical. This was done by both the procedures that have been used for homologous preparations with lysine, i.e. (1) through the NN^{ϵ}-dicarbobenzoxy derivative, its acid chloride and the cyclic α-*N*-carboxy anhydride (Bergmann, Zervas & Ross, 1935); (2) through the copper complex of the free amino-acid (Neuberger & Sanger, 1943; cf. Kurtz, 1938). Sanger (1946) has already employed the latter procedure for the preparation of δ-*N*-2:4-dinitrophenylornithine. Both procedures gave similar yields, but the copper method involved somewhat simpler manipulations. The fact that the monocarbobenzoxyornithine obtained reacted with ninhydrin to yield one molecule of CO_2 provided further evidence that it was the δ-carbobenzoxy derivative. Van Slyke, Dillon, MacFadyen & Hamilton (1941) have demonstrated that ε-carbobenzoxylysine yields one molecule of CO_2 with ninhydrin.

The advantages of using dilute anhydrous HCl in cold methanol for esterifying proteins and other compounds somewhat readily split by acid, such as *N*-acylamino-acids, have been demonstrated by Fraenkel-Conrat & Olcott (1945). The present work has shown that the method is applicable to carbobenzoxy derivatives of amino-acids (δ-carbobenzoxyornithine, carbobenzoxyvaline) and to free amino-acids (valine, leucine).

The purity of the resulting dipeptides has been checked in every case by paper-strip chromatograms with a suitable solvent system (cf. Consden, Gordon & Martin, 1944). In some preliminary experiments the products, when tested in this way, showed, in addition to the desired compound, a complex array of contaminants that coloured with ninhydrin. Such results occurred in the syntheses of valylornithine and leucylphenylalanine, if the coupling was effected with acid chloride that had been purified in the usual way by washing with ligroin; in these cases the treatment appears inadequate to remove phosphorus compounds that may have deleterious effects. It was found that washing of the acid chlorides with water, as in the preparation of carbobenzoxyleucyl chloride by Bergmann, Zervas & Fruton (1936), gave much purer products. Good results were also obtained in this way in the synthesis of prolylvaline. It appears, as pointed out to me by Dr C. R. Harington, that these acid chlorides are much less sensitive to moisture than would appear from the literature. It was also found that the coupling reactions, in the syntheses of valylornithine and leucylphenylalanine, proceeded much more rapidly, giving better yields, in organic solvents shaken with saturated aqueous $KHCO_3$, than in dry mixtures of organic solvents. This procedure also effects a valuable economy in the amount of ester that has to be used.

A point worth re-emphasizing is the importance of the general practice of employing amino-acid esters rather than free amino-acids in the coupling. In the present work, in view of the reported instability of proline ester to moisture (Abderhalden & Sickel, 1926), and following the procedure of Bergmann, Zervas, Schleich & Leinert (1932) for making peptides of proline, free proline was employed for the synthesis of phenylalanylproline. The separation of the desired carbobenzoxydipeptide from carbobenzoxyamido-acid resulting from decomposition of the acid chloride with water is then difficult. Furthermore, the free carboxyl groups of free amino-acid and/or carbobenzoxydipeptide may react with unchanged acid chloride to form mixed anhydrides, which in turn can react with more amino-acid, and so on, to form a complicated mixture of peptide derivatives. Both these difficulties are avoided by adhering to the use of the esters for coupling, and, in view of the impurity associated with the product of the phenylalanylproline synthesis described here, it would be worth searching for a derivative of proline for such syntheses in which the carboxyl group is blocked by a suitable radical. A similar formation of mixed anhydrides of acetic acid with acetamido-acids may explain the difficulty of quantitatively converting the mixture of amino-acids in a protein hydrolysate to acetamido-acids for chromatographic analyses (Gordon, Martin & Synge, 1943;

Tristram, 1946). Some of the 'artefacts' observed in such analyses, on this view, might be acetyl-peptides.

EXPERIMENTAL

General technical notes

Unless otherwise stated, compounds were recrystallized before analysis until the recorded m.p. and/or optical rotation (usually both) were substantially constant. M.p.'s are given uncorrected. Optical rotations were determined in a 0·5 dm. tube. Evaporations *in vacuo* were conducted below 40° unless otherwise stated.

One-dimensional paper partition chromatograms were done according to Consden *et al.* (1944). The R_F values observed for the dipeptides are given by Consden *et al.* (1947).

Nitrogen determinations were made by a micro-Kjeldahl procedure. For the estimation of the acid equivalent weight (AEW), the sample was titrated in aqueous ethanol with 0·01 N-$Ba(OH)_2$, employing phenolphthalein as internal indicator. The titration mixture was subsequently used for N determination. C, H and Cl (Carius) determinations were by Dr G. Weiler, Oxford.

Catalytic reduction of carbobenzoxy compounds was effected in the usual way; 50–250 mg. of the carbobenzoxy compound were dissolved in 1 ml. glacial acetic acid, 10 ml. methanol and 1–2 ml. water, and hydrogenated at atmospheric pressure and room temperature in the presence of 0·1–0·2 g. active Pd (Willstätter & Waldschmidt-Leitz, 1921). In the case of the carbobenzoxy derivatives of the ornithine peptides, 2–3 mol. equiv. of HCl were added in the water, and in the preparation of ornithylleucine 10 ml. acetic acid and no methanol were arbitrarily employed in the solvent mixture. In every case CO_2 evolution was complete after 0·5–1 hr.

Starting materials. The amino-acids employed for the present syntheses were prepared in this laboratory, and tested for the absence of expected contaminant amino-acids with suitable solvents on paper partition chromatograms. Stereochemical purity was checked in each case by determinations of optical rotation of the amino-acid or its formyl derivative.

The racemic synthetic amino-acids were resolved through their formyl derivatives by the methods of Fischer (1906). After characterization, the formyl derivatives were hydrolyzed with hydrochloric acid (the strength of acid and duration of refluxing being as given by Fischer), and the hydrolysate was evaporated to dryness *in vacuo* up to 100° several times with water. The resulting amino-acid hydrochlorides were used directly for the syntheses.

L-*Ornithine monohydrochloride.* L-Arginine monohydrochloride was prepared from a HCl hydrolysate of gelatin (Cox, 1928). From this, L-ornithine monohydrochloride ($[\alpha]_D^{18^\circ}$ + 10·8° (water, $c=5$)) was prepared by the arginase method of Hunter (1939).

L-*Proline.* The above gelatin hydrolysate, freed from arginine by flavianic acid, was subjected to the rhodanilate procedure of Bergmann (1935) for the isolation of L-proline. The product, dried to constant weight at 60° over P_2O_5 *in vacuo* had $[\alpha]_D^{19^\circ}$ − 81·9° (water, $c=7·0$).

L-*Valine.* *iso*Propanol was converted to *iso*propyl bromide (Norris, 1907) and this, by malonic ester synthesis (Marvel & du Vigneaud, 1943) to α-bromo*iso*valeric acid, which was aminated (Marvel, 1940) to DL-valine. This was resolved (Fischer, 1906) yielding formyl-L-valine, m.p. 151–152°, $[\alpha]_D^{18^\circ}$ − 16·6° (water, $c=5·0$).

L-*Leucine.* *iso*Butyl bromide, by malonic ester synthesis (Marvel & du Vigneaud, 1943) yielded α-bromo*iso*caproic acid which was aminated (Marvel, 1941) to DL-leucine. This was resolved (Fischer & Warburg, 1905) yielding formyl-L-leucine, m.p. 137–140°, $[\alpha]_D^{18^\circ}$ − 18·6 (ethanol, $c=8·7$).

D-*Phenylalanine.* DL-Phenylalanine was prepared from hippuric acid and benzaldehyde (Gillespie & Snyder, 1943). It was resolved (Fischer & Schoeller, 1907) to yield formyl-D-phenylalanine, m.p. 164–166°, $[\alpha]_D^{18^\circ}$ − 74·5° (ethanol, $c=3·3$).

Substitution products of ornithine used as intermediates

(*a*) *δ-Carbobenzoxy-*L*-ornithine methyl ester through the* Cu *derivative of ornithine.* A solution of L-ornithine monohydrochloride (5 g.) in 50 ml. water was saturated with $CuCO_3$ at 100°, cooled and filtered. The resulting deep-blue solution was treated with 2 N-NaOH and benzyl chloroformate, added alternately in 6 equal portions over a period of 20 min. at 0°, whilst the flask was vigorously shaken. In all, 30 ml. 2 N-NaOH and 6 ml. benzyl chloroformate were added. The mixture was then shaken mechanically at room temperature for 30 min. The pale-blue precipitate was then filtered off and thoroughly washed on the filter, first with water, and then with ethanol. The air-dry precipitate was suspended in 150 ml. water, and treated with H_2S overnight in a closed vessel. Water (550 ml.) was then added, the mixture was boiled for a few minutes and rapidly filtered from CuS through a pre-heated filter. The CuS was further washed with 300 ml. of boiling water. On cooling the combined filtrate and washings, *δ-carbobenzoxy-*L*-ornithine* (3 g.) crystallized out in needles. The mother liquors yielded on concentration *in vacuo* only 0·3 g. of inferior material which was not utilized. $[\alpha]_D^{16^\circ}$ + 17° (in 1 : 1 v/v aqueous acetone in the presence of 2 mol. HCl, $c=2·9$). Found: C, 58·8; H, 7·3; N, 10·5. $C_{13}H_{18}O_4N_2$ requires C, 58·6; H, 6·8; N, 10·5%. A ninhydrin-CO_2 determination (Van Slyke *et al.* 1941) at a total volume of 1·25 ml., pH 2·5, with 50 mg. ninhydrin gave carboxyl N, 5·2%. (Theory requires 5·3%.)

2 g. δ-Carbobenzoxy-L-ornithine were dissolved in 100 ml. anhydrous methanol, N in respect of HCl, and kept for 24 hr. at room temperature. The mixture was evaporated to dryness *in vacuo*. After two further such treatments with methanolic HCl, the residue crystallized rapidly and completely. It was recrystallized from hot acetone, ether being added to increase the completeness of recovery. The yield of *δ-carbobenzoxy-*L*-ornithine methyl ester hydrochloride* was 2·35 g. For characterization see (*c*) below.

(*b*) *αδ-Dicarbobenzoxy-*L*-ornithyl chloride, and δ-carbobenzoxy-*L*-ornithine methyl ester therefrom.* 1·25 g. L-ornithine monohydrochloride were dissolved in 8 ml. 2 N-NaOH and to the mixture, cooled to 0°, were added alternately, with shaking, four 4·5 ml. portions of 2 N-NaOH and four 0·9 ml. portions of carbobenzoxy chloride. After some minutes the mixture was acidified with 10 N-HCl (5·5 ml.). The oil which was precipitated was taken up in 25 ml. ether, and the ether extract was counter-extracted with four successive 50 ml. portions of 7% (w/v) $KHCO_3$ solution. The combined

$KHCO_3$ extracts were acidified with 25 ml. 10 N-HCl, and extracted with ether containing some ethyl acetate. The organic solvent layer was after drying evaporated to dryness *in vacuo*. The syrupy residue (2·7 g.) was crystallized from ether-light petroleum. The product was filtered off, washed with light petroleum and dried (2·47 g.). *αδ-Dicarbobenzoxy-L-ornithine* was recrystallized for analysis from ether-light petroleum, and had m.p. 112–114°, $[\alpha]_D^{20^\circ}$ $-4°$ (ethanol, $c=3$). Found: C, 62·6; H, 6·1; N, 6·8; AEW, 376. $C_{21}H_{24}O_6N_2$ requires C, 63·0; H, 6·0; N, 7·0%; AEW, 400.

0·64 g. αδ-Dicarbobenzoxy-L-ornithine and 0·35 g. PCl_5 were pulverized separately and then shaken together in dry ether (12 ml.) in a glass-stoppered vessel at 5–10°. Before all of the reactants had dissolved, a fluffy crystalline precipitate of *αδ-dicarbobenzoxy-L-ornithyl chloride* had begun to form. When no further obvious reaction was occurring, the mixture was cooled to 0° for 1 hr., with intermittent shaking. Dry ligroin (8 ml.) was then added, and the mixture was kept at 0° for a further hour. The crystals were then filtered off and dried. Yield, 0·63 g. of material having m.p. (sealed tube) 54–56°. The product was not analyzed, and was used immediately in the synthesis of ornithylleucine.

For the preparation of δ-carbobenzoxyornithine derivatives, as in the preparation of ϵ-carbobenzoxylysine derivatives (Bergmann *et al.* 1935), it was not necessary to isolate the acid chloride. A preparation was made as above from 0·84 g. αδ-dicarbobenzoxy-L-ornithine. Instead, however, of adding ligroin to the reaction mixture, some dry ethyl acetate was added, whereupon the crystals dissolved. The mixture was evaporated to dryness at room temperature, yielding a crystalline residue. On heating *in vacuo* to 60° the crystals melted, and the resulting syrup gassed freely. It was dissolved in dry ethyl acetate and evaporated to dryness *in vacuo* twice (odour of benzyl chloride) and further heated for 1 hr. at 40–50°. The syrupy residue was then dissolved in ethyl acetate and brought to crystallization by addition of ether and light petroleum. After one recrystallization, 0·45 g. of product resulted. *δ-Carbobenzoxy-L-ornithine-α-N-carboxy anhydride* had m.p. 86–88°, $[\alpha]_D^{19^\circ}$ $-18°$ (ethyl acetate, $c=4$). Found: C, 57·2; H, 6·0; N, 9·3. $C_{14}H_{16}O_5N_2$ requires C, 57·5; H, 5·5; N, 9·6%. The compound appeared more stable than the corresponding lysine derivative (Bergmann *et al.* 1935), since after storage in a desiccator for 6 months the appearance and m.p. were unchanged.

0·4 g. of the anhydride was dissolved in 15 ml. anhydrous methanol, N in respect of HCl. Immediate evolution of gas was noticed. The mixture was warmed to 50°, and then kept overnight at room temperature. On evaporation *in vacuo* the product crystallized. Recrystallization from acetone-ether yielded 0·34 g. of *δ-carbobenzoxy-L-ornithine methyl ester hydrochloride*. The properties and analytical data are given below.

(*c*) *Comparison of δ-carbobenzoxy-L-ornithine methyl ester hydrochloride prepared by the two methods.* A comparison of specimens obtained by procedures (*a*) and (*b*) above gave the following results. M.p. (*a*) 132–134°; (*b*) 132–134°; mixture, 132–134°. $[\alpha]_D^{19}$ (*a*) +14°, +15°, (*b*) +13° (methanol, $c=4$). Found: C, (*a*) 52·6, (*b*) 52·9; H, (*a*) 6·6, (*b*) 6·5; N, (*a*) 8·8, (*b*) 8·5; Cl, (*a*) 13·1, (*b*) 11·1%. $C_{14}H_{20}O_4N_2$.HCl requires C, 53·1; H, 6·6; N, 8·8; Cl, 11·2%.

α-(L-Valyl)-L-ornithine

The evaporated hydrolysate from 1·9 g. formyl-L-valine (see above) was dissolved in 2 N-NaOH (16 ml.) and carbobenzoxylated as above using in all 8·25 ml. 2 N NaOH and 2·6 ml. benzyl chloroformate. The mixture was shaken at room temperature for a further 20 min. and acidified with 4 ml. 10 N-HCl. The oil which precipitated was extracted into about 20 ml. ether, and the ether extract was counter-extracted with five successive 16 ml. portions of 7% (w/v) $KHCO_3$ solution. The combined $KHCO_3$ extracts were acidified with 10 N-HCl, and extracted with an equal volume of ether. The ether extract was evaporated *in vacuo* (50°) to a clear syrup (2·6 g.). After some weeks of storage in a desiccator, the syrup crystallized, and after recrystallization from ether-light petroleum, *carbobenzoxy-L-valine* was found to have m.p. 64–65°, $[\alpha]_D^{20^\circ}$ $+4°$ (ethanol, $c=2·9$). Found: C, 62·6; H, 7·0; N, 5·5; AEW, 251. $C_{13}H_{17}O_4N$ requires C, 62·2; H, 6·8; N, 5·6%; AEW, 251. The methyl ester was prepared from carbobenzoxy-L-valine by treatment with methanolic HCl. 0·38 g. of syrupy carbobenzoxy-L-valine was dissolved in 15 ml. methanol N in respect of HCl, and kept at room temperature for 24 hr. At the end of this time the mixture was evaporated to dryness *in vacuo*: the whole treatment was repeated and the residue was dissolved in ether and water. The ether layer was separated, washed with $KHCO_3$ solution, then with water, and evaporated to dryness *in vacuo*. The product (0·39 g.) crystallized readily, and was purified by recrystallization from ether-light petroleum. *Carbobenzoxy-L-valine methyl ester* was found to have m.p. 56–57°, $[\alpha]_D^{17^\circ}$ $-16°$ (ethanol, $c=3·1$). Found: C, 63·6; H, 7·2; N, 5·1. $C_{14}H_{19}O_4N$ requires C, 63·4; H, 7·2; N, 5·3%.

Carbobenzoxy-L-valyl chloride was frequently obtained crystalline on evaporating *in vacuo* the product of reaction of equimolecular quantities of carbobenzoxy-L-valine and PCl_5 in dry ether. It was not, however, characterized, and was best used for the coupling with δ-carbobenzoxy-L-ornithine methyl ester without isolation, as follows. 0·77 g. carbobenzoxy-L-valine was dissolved in 8 ml. ether (dried over Na) and 0·75 g. powdered PCl_5 was added. The temperature was maintained initially at 0°, but as solution of the PCl_5 occurred rather slowly, the mixture was allowed to approach room temperature. When the PCl_5 was completely or nearly dissolved, the mixture was diluted with a further 8 ml. ether and quickly washed twice with equal volumes of ice water. The ether layer was quickly treated with Na_2SO_4 and the solution was then mixed with 30 ml. of a solution of δ-carbobenzoxy-L-ornithine methyl ester in ethyl acetate. (The ester was liberated from 1·25 g. of its hydrochloride with K_2CO_3 in the usual way into ether; after evaporation *in vacuo* the syrupy ester was dissolved in ethyl acetate.) To the reaction mixture were added 7 ml. of a saturated solution of $KHCO_3$. The mixture was kept at room temperature and shaken at intervals over a period of 2 hr. Water was then added, and the organic solvent layer was washed successively with water, N-HCl, water, 7% (w/v) $KHCO_3$, and water, partially dried with Na_2SO_4, and evaporated to dryness *in vacuo*, giving 0·55 g. of a colourless, crystalline residue smelling of benzyl chloride. Recrystallization from chloroform-light petroleum yielded 0·41 g. of material satisfactory for further work. *α-(Carbobenzoxy-L-valyl)-δ-carbobenzoxy-L-ornithine methyl ester* had m.p. 155–156°, $[\alpha]_D^{18°}$ ±0° (B.P. chloroform, $c=2·5$). Found: C, 62·7; H, 6·9; N, 8·1. $C_{27}H_{35}O_7N_3$ requires C, 63·2; H, 6·8; N, 8·2%.

0·20 g. of this compound was saponified by dissolving in 3 ml. dioxan and adding 2 ml. N-NaOH. Crystals precipitated, but these dissolved on gently shaking for 10 min. After a further 30 min. the mixture was diluted with water to about 50 ml. On acidification with 0·4 ml. 10 N-HCl an oil was precipitated, which crystallized rapidly. Yield, 0·19 g. *α-(Carbobenzoxy-L-valyl)-δ-carbobenzoxy-L-ornithine* was recrystallized from 20 parts of methanol by gradual addition of water and had m.p. 193–195°, $[\alpha]_D^{20°}$ −11° (glacial acetic acid, $c=0·9$). Found: C, 62·5; H, 6·6; N, 8·3; AEW, 504. $C_{26}H_{33}O_7N_3$ requires C, 62·5; H, 6·6; N, 8·4%; AEW, 499.

0·15 g. was hydrogenated as described above. The syrupy residue was kept in a high vacuum in a desiccator over soda lime, dissolved in aqueous ethanol, and neutralized to bromothymol blue with NH_3. The mixture was evaporated *in vacuo* to dryness and treated with ethanol. Crystallization resulted, yielding *α-(L-valyl)-L-ornithine monohydrochloride (hydrated)* (0·08 g.) which was recrystallized from small volumes of water by addition of much ethanol. The optical rotation before and after recrystallization was respectively $[\alpha]_D^{19°}$ +18° and +19° (water, $c=2$). The recrystallized material showed two very faint contaminants on a paper-strip chromatogram developed with *s*-collidine, one moving faster, and the other slower than the principal zone. The product was deliquescent, and was stored in a sealed tube. The rotation determination and analyses are referred to material dried in a vacuum desiccator over H_2SO_4-soda lime. Found: C, 43·8; H, 8·1; N, 15·0; amino N (Van Slyke, 0·5 hr. reaction time), 10·8; Cl, 13·9. $C_{10}H_{21}O_3N_3.HCl.\frac{1}{2}H_2O$ requires C, 43·4; H, 8·3; N, 15·2; amino N, 10·1; Cl, 12·8%.

L-*Ornithyl*-L-*leucine*

L-Leucine methyl ester hydrochloride (Abderhalden & Spinner, 1919; cf. Smith & Brown, 1941) was prepared in nearly theoretical yield from L-leucine by the same method as used for the preparation of δ-carbobenzoxy-L-ornithine methyl ester. 0·58 g. of the ester hydrochloride was decomposed with K_2CO_3 in the usual way. To the dry ethereal solution of the free ester (20 ml.) at 0° was added a solution of 0·63 g. αδ-dicarbobenzoxy-L-ornithyl chloride in 5 ml. ethyl acetate. Immediate separation of needles of leucine methyl ester hydrochloride resulted. The mixture was stored overnight at 0°; the crystals were then filtered off, and the filtrate was washed successively with water, N-HCl, 7% (w/v) $KHCO_3$ solution (twice) and water, and evaporated to dryness *in vacuo*. The syrupy residue gave crystals from chloroform-light petroleum (0·66 g.). *αδ-Dicarbobenzoxy-L-ornithyl-L-leucine methyl ester* was recrystallized from ethyl acetate-ether-light petroleum, and had m.p. 83–84°, $[\alpha]_D^{20°}$ −18° (ethanol, $c=2·8$). Found: C, 63·7; H, 7·1; N, 7·9. $C_{28}H_{37}O_7N_3$ requires C, 63·8; H, 7·0; N, 8·0%. 0·57 g. of this compound was dissolved in 2·1 ml. dioxan and saponified by addition of 2·1 ml. N-NaOH. The mixture was kept for 0·5 hr. after becoming homogeneous and was then diluted with water to 35 ml. On acidifying with 10 N-HCl oil was precipitated, which soon crystallized, yielding 0·535 g. of crystalline material, having m.p. 60–61°, and suitable for the next step. The material was *αδ-dicarbobenzoxy-L-ornithyl-L-leucine* crystallizing, according to the analytical data, with 0·5 mol. dioxan, not removable by drying *in vacuo* at temperatures below the m.p. Recrystallization from methanol-H_2O yielded hydrated material from which the water could likewise not be removed at temperatures below the m.p. This material, dried in a desiccator at room temperature, had m.p. 63–64°, $[\alpha]_D^{17°}$ −14° (methanol, $c=4·2$). Found: C, 59·4; H, 6·8; N, 7·6; AEW, 527. $C_{27}H_{35}O_7N_3.2H_2O$ requires C, 59·0; H, 7·1; N, 7·7%; AEW, 549.

0·5 g. (with dioxan of crystallization) was hydrogenated as described above. The product, a colourless gum, was dissolved in water (slight turbidity) and the solution was neutralized to bromothymol blue by addition of ammonia. The mixture was again evaporated to dryness *in vacuo*, and on treatment with ethanol yielded 0·125 g. of crystals, which were recrystallized from very concentrated aqueous solution by addition of ethanol. The analytical data suggest that the product was *L-ornithyl-L-leucine monohydrochloride* incorporating half a molecule of water of crystallization and somewhat contaminated with the dihydrochloride. It was not deliquescent, and showed on a paper chromatogram developed with *s*-collidine a very faint trace of contaminant material running just ahead of the main component. $[\alpha]_D^{18°}$ +2° (water, $c=2·1$). Found: C, 45·2; H, 8·3; N, 14·2; amino N (Van Slyke, 0·5 hr. reaction time), 9·5; Cl, 14·3. $C_{11}H_{23}O_3N_3.HCl.\frac{1}{2}H_2O$ requires C, 45·4; H, 8·6; N, 14·5; amino N, 9·7; Cl, 12·2%.

L-*Leucyl*-D-*phenylalanine*

From carbobenzoxy-L-leucine was prepared the acid chloride (Bergmann *et al.* 1936) by treating 0·25 g. with 0·25 g. PCl_5 in dry ether at 0°. When reaction was complete, the mixture was twice washed rapidly with equal volumes of cold water, and dried over Na_2SO_4. To this solution at 0° was added D-phenylalanine ethyl ester (prepared from 0·25 g. of its hydrochloride) in 4 ml. ethyl acetate. 3 ml. saturated aqueous $KHCO_3$ were added, and the mixture was kept at 0° with intermittent shaking for 1 hr. Water was added, and the organic solvent layer was separated and washed successively with N-HCl and 7% (w/v) $KHCO_3$ solution, dried, and concentrated *in vacuo*. The residue crystallized; recrystallization from ether-light petroleum yielded 0·22 g. *carbobenzoxy-L-leucyl-D-phenylalanine ethyl ester* suitable for further work. The product was recrystallized from chloroform-light petroleum, and had m.p. 103–105°, $[\alpha]_D^{17°}$ −19° (ethanol, $c=2·7$). Found: C, 67·9; H, 7·1; N, 6·3. $C_{25}H_{32}O_5N_2$ requires C, 68·2; H, 7·3; N, 6·4%.

0·2 g. of this compound was dissolved in 1·5 ml. dioxan, and 1·5 ml. N-NaOH were added. The mixture, which became homogenous on gentle shaking, was kept for 45 min. at room temperature, and was then largely diluted with water and acidified. The oil which was precipitated was isolated by extraction with chloroform. The chloroform extract was evaporated to dryness *in vacuo*, yielding a syrupy residue of *carbobenzoxy-L-leucyl-D-phenylalanine*. This did not readily crystallize and was hydrogenated as described above. The gummy product was dissolved in 95% (v/v) aqueous ethanol; the solution was concentrated *in vacuo* until crystallization began, and ether was then added. In this way was obtained 0·12 g. of *L-leucyl-D-phenylalanine dihydrate*. A paper chromatogram with *n*-butanol showed only one band colouring with ninhydrin.

The product was recrystallized from warm 50% (v/v) aqueous ethanol. The water of crystallization was not readily lost in a vacuum desiccator at room temperature, but was removed on drying at 100° over P_2O_5 *in vacuo*. The hydrated material had $[\alpha]_D^{17^\circ}$ +25° (glacial acetic acid, $c=2\cdot4$) and was employed for analysis. Found: C, 57·3; H, 7·9; N, 8·7; loss of weight on drying, 11·7. $C_{15}H_{22}O_3N_2 . 2H_2O$ requires: C, 57·3; H, 8·3; N, 8·9; loss of weight on drying, 11·5%. An amino-N determination (Van Slyke, 4 min. reaction time) on the material dried at 100° gave amino N, 5·0%; $C_{15}H_{22}O_3N_2$ requires 5·0%. Thus the water lost was solvent of crystallization, and cyclization to the diketopiperazine had not occurred.

D-*Phenylalanyl*-L-*proline*

0·4 g. of carbobenzoxy-D-phenylalanine was prepared from D-phenylalanine and converted to the acid chloride according to Smith & Brown (1941). The product was washed by decantation with dry ligroin and allowed to react with 0·3 g. L-proline dissolved in 4·4 ml. N-NaOH (cf. Bergmann *et al.* 1932). After 20 min. shaking at room temperature the still alkaline mixture was filtered, and the filtrate acidified (thymol blue) with 10N-HCl. An oil was precipitated, which hardened on keeping at 0° for a few hours. This material, presumed to be largely *carbobenzoxy-D-phenylalanyl-L-proline*, was filtered off and washed with water. Yield, 0·13 g. On attempting recrystallization, only rather greasy crystals were obtained. Accordingly, the whole was hydrogenated as described above. The reaction product was dissolved in ethanol. On addition of ether a flocculent precipitate resulted, which was filtered off, washed with ether, dried, and reprecipitated from the same solvents. The preparation had approximately the same optical rotation ($[\alpha]_D^{18^\circ}$ -93° and -96°, respectively, (acetic acid, $c=2\cdot4$)) before and after reprecipitation. The final amorphous product (0·07 g.) proved, however, on paper chromatography with *n*-butanol, to be complex, giving a well marked faster moving band in addition to the stronger band corresponding to D-*phenylalanyl*-L-*proline*. The elementary analyses likewise depart considerably from the expected values; some of this deviation may, however, be due to retention of ethanol by the amorphous material dried at room temperature in a vacuum desiccator. Found: C, 62·4; H, 7·0; N, 8·3. $C_{14}H_{18}O_3N_2$ requires C, 64·1; H, 6·9; N, 10·7%. An attempt was made to prepare the acetyl derivative (cf. Behrens, Doherty & Bergmann, 1940) by acetylation with acetic anhydride and aqueous NaOH, in the usual way. Extraction of the acidified reaction mixture with *n*-butanol in chloroform (17% v/v), did not, however, yield satisfactory crystals.

L-*Prolyl*-L-*valine*

L-*Valine methyl ester hydrochloride* was prepared in nearly theoretical yield from L-valine by the methanolic HCl procedure used above. The reaction product crystallized on evaporation to dryness *in vacuo* and was recrystallized first from acetone-ether and subsequently from methanol-ether. The product, resulting in nearly theoretical yield, had m.p. 146–149°, $[\alpha]_D^{20^\circ}$ +14° (water, $c=3\cdot0$) after drying in a vacuum desiccator at room temperature. Lower, and less sharp m.p.'s were observed with air-dry material, indicating the presence of solvent of crystallization. That some HCl, present as such, was incompletely removed even in the desiccator, is indicated by the analytical data for desiccator-dried material. Found: C, 41·8; H, 8·3; N, 8·2; Cl, 24·7. $C_6H_{13}O_2N . HCl$ requires C, 43·0; H, 8·4; N, 8·4; Cl, 21·2%.

Carbobenzoxy-L-proline was prepared according to Abderhalden & Nienburg (1933) from L-proline in 75% of the theoretical yield. 0·5 g. of the syrupy carbobenzoxy-L-proline was dissolved in 5 ml. dry ether and 0·5 g. PCl_5 was added to this solution at 0°. After shaking gently for 15 min. most of the PCl_5 had dissolved. The ethereal solution was decanted from undissolved PCl_5, and washed twice, quickly, with ice water and partially dried. To this solution was added an ethyl acetate solution of L-valine methyl ester, liberated from 0·4 g. of its hydrochloride by aqueous K_2CO_3. Saturated aqueous $KHCO_3$ was added to the mixture which was shaken intermittently during 20 min. After a further 40 min. water was added and the organic solvent layer was washed successively with N-HCl and 7% (w/v) aqueous $KHCO_3$, partially dried, and evaporated *in vacuo*, yielding 0·55 g. of colourless syrup. Attempts to bring this material, *carbobenzoxy-L-prolyl-L-valine methyl ester*, to crystallization were unsuccessful. 0·45 g. was saponified in 3 ml. dioxan with 3 ml. N-NaOH. The mixture quickly became homogeneous, and after 0·5 hr. at room temperature, was diluted with water. On acidifying (HCl), an oil was precipitated. This crystallized within a few hours. Yield, 0·33 g. of material suitable for subsequent use. *Carbobenzoxy-L-prolyl-L-valine* was recrystallized from methanol-water, and had m.p. 134–135°, $[\alpha]_D^{18^\circ}$ -58° (methanol, $c=2\cdot8$). Found: C, 62·5; H, 6·9; N, 8·0; AEW, 350. $C_{18}H_{24}O_5N_2$ requires C, 62·1; H, 6·9; N, 8·0%; AEW, 348.

0·18 g. was hydrogenated as described above. 0·10 g. of crystalline material resulted. A paper-strip chromatogram developed with *n*-butanol revealed, on colouring with ninhydrin, a single yellow band. The material was recrystallized from water by addition of ethanol. Analysis showed the product to be L-*prolyl*-L-*valine hydrate*. The water of crystallization was lost very slowly in a vacuum desiccator at room temperature. $[\alpha]_D^{20^\circ}$ -61° (water, $c=1\cdot7$) (calc. for the hydrate). The air-dry hydrate was used also for the analyses. Found: C, 52·1; H, 8·5; N, 12·6; amino N (Van Slyke, 4 min. reaction time), 0·0. $C_{10}H_{18}O_3N_2 . H_2O$ requires C, 51·7; H, 8·6; N, 12·1%; amino N, nil.

SUMMARY

1. Syntheses are described of the dipeptides α-(L-valyl)-L-ornithine, L-ornithyl-L-leucine, L-leucyl-D-phenylalanine, D-phenylalanyl-L-proline and L-prolyl-L-valine. Carbobenzoxy derivatives were employed as intermediates, coupling being effected in each case through an acid chloride.

2. Some factors affecting the purity of the product in such syntheses are discussed.

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A Method for Estimating Peptic Activity in Gastric Contents

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An accurate simple method of estimating the activity of proteolytic enzymes is clearly desirable. To achieve this end, not only must a precise method of measuring proteolysis be devised, but a reproducible substrate must also be found. These two requirements were first met in the method of estimating pepsin described by Anson & Mirsky (1932) and Anson (1938). Their method, in which the substrate is carboxyhaemoglobin, has been widely used for estimating pure enzymes on account of its accuracy. In modified forms it has proved valuable in the determination of the activity of digestive juices (Beazell, Schmidt, Ivy & Monoghan, 1938; Bucher, Grossman & Ivy, 1945). However, without large scale apparatus, the preparation of sufficient quantities of the substrate of carboxyhaemoglobin is time-consuming and the method is correspondingly restricted in its applicability.

This paper describes the use of another reproducible but more readily available substrate. The modified reagents and procedure are given in full. A method of standardization is described and the adoption of a new pepsin unit is suggested.

METHOD

The substrate

General remarks. Dehydrated human plasma and serum, rejected as unfit for transfusion purposes, have been used exclusively for this work. The dried plasma or serum is of variable colour and is freely soluble in distilled water. Two samples have been encountered which immediately formed a gel on addition to water. These were discarded.

The substrate solution. Substrate solution is made up to contain 5·6 g. dried citrated plasma or dried serum in 100 ml. of distilled water with sufficient HCl to give an acidity of pH 2·1 as determined with a glass electrode at 18–22°. The substrate solution is stirred and then filtered through a Green's 309½ Agar filter paper or cotton wool. Considerable variations in the rate of filtration have been encountered but this had no apparent significance in the method.

Other reagents are: 0·350 N-trichloroacetic acid (approximately 6 g./100 ml.), 0·250 N-NaOH, HCl solution, pH 2·1, Folin & Ciocalteu's (1927) phenol reagent, phenol solution (5·0 mg./100 ml.).

Estimation of proteolytic activity

The sample of gastric juice under examination is mixed with an equal volume of dilute HCl of pH 2·1. A series of pairs of test tubes, one tube containing 5·0 ml. of substrate and the other 10·0 ml. trichloroacetic acid, are placed in a water bath at 35°. Stock tubes of gastric juice mixtures are also warmed in the water bath. After 20 min. 1·0 ml. of gastric juice mixture is added to the substrate tubes of pairs 1, 3, 5 *et seq.* and to the trichloroacetic acid tubes of pairs 2, 4, 6 *et seq.* Each addition is made at regular intervals of 30 sec. so that a series of up to 15 estimations can be handled in 14 min. Mixing is ensured by agitation of the tubes outside the water bath. Thus peptic digestion proceeds in the substrate tubes of the odd-numbered pairs whilst the even pairs provide controls.

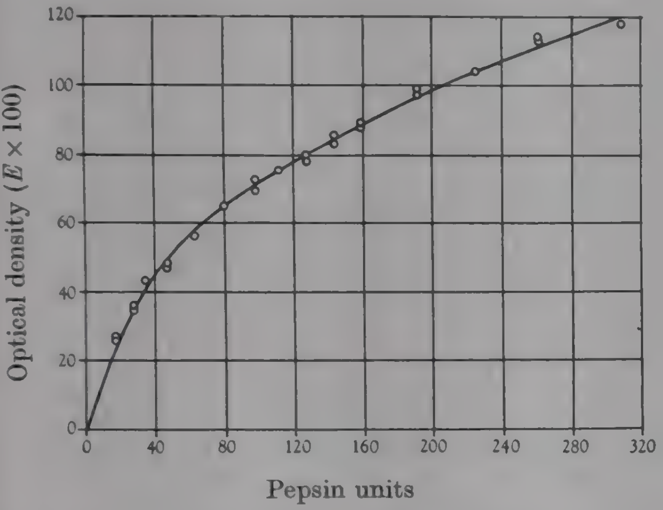

Fig. 1. The relationship between the difference in colour between control and test and the peptic activity of the gastric juice.

Exactly 15 min. after the addition of gastric juice to any pair of tubes, their contents are mixed by pouring back and forth. This stops any digestion in the odd-numbered pairs. After this mixing the tubes are allowed to stand in the water bath for approximately 4 min. The mixture is filtered through a 15 cm. paper (Whatman no. 1). 15–30 min. after the beginning of filtration 2·0 ml. of the filtrate are pipetted into a 50 ml. flask containing 20 ml. 0·25 N-NaOH and thorough mixing is ensured by swirling (vigorous shaking must be avoided as it tends to reduce the development of colour). Forthwith 1·0 ml. of Folin & Ciocalteu's phenol reagent is run down the side of the flask and mixed by immediate swirling.

A blue colour gradually develops and 15–40 min. later it is estimated in a photoelectric colorimeter with a suitable filter. A single cell colorimeter (King, 1946) with an Ilford 204 filter has been found satisfactory.

The difference in colour between the test and the control is used to evaluate the peptic activity from a curve which relates activity to colour difference (Fig. 1). If a more accurate evaluation is required and the peptic activity falls between 25 and 60 pepsin units/ml. (see below), the straight-line relationship, in which the peptic activity is plotted on a logarithmic scale can be used (Fig. 2). These curves can be plotted from the values shown in Table 1.

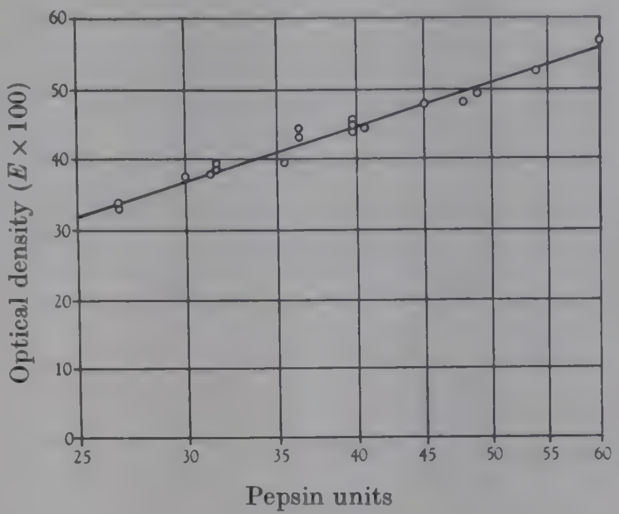

Fig. 2. The relationship between the difference in colour between control and test and the peptic activity of the gastric juice, plotted on a logarithmic scale.

Table 1. *Relation of peptic activity of gastric juice to difference in colour between control and test*

Pepsin units	Optical density ($E \times 100$) (Difference between control and test)
10	15·0
15	21·0
20	27·0
25	32·0
30	36·8
35	41·0
40	44·5
45	48·0
50	51·0
60	56·0
70	60·5
80	64·5
90	68·0
100	71·4
110	75·0
120	78·0
130	81·0
140	83·5
150	86·2
160	89·0
180	94·0
200	98·7
220	103·0
240	107·2
260	111·5
280	115·0
300	118·0

A gastric juice is said to have 50 units of peptic activity/ml. when under the standard conditions of estimation (viz. after dilution with an equal part of HCl solution of pH 2·1) it gives a colour equivalent to that produced by 2·0 ml. of a standard phenol solution (5·0 mg./100 ml.).

The standard colour is developed by mixing 2·0 ml. of freshly prepared phenol solution (5·0 mg./100 ml.) with 20·0 ml. 0·25 N-NaOH and promptly adding 1·0 ml. Folin & Ciocalteu's phenol reagent. Between 5–10 min. later the colour is read in the colorimeter. A blank estimation is made omitting the phenol; in this way ten consecutive estimations gave values, after subtracting the blanks, of 0·51 with no measurable deviations.

The relation of peptic activity to the colour produced

Since the method was devised to determine the proteolytic activity of gastric juice, this was used in preference to a solution of pure pepsin in establishing the relationship between proteolytic activity and colour differences between control and test. A series of known dilutions of a gastric juice were made. After 15 min. digestion, the value of the difference in colour between control and test for each of these dilutions was recorded. With these values as ordinates and percentage concentration of gastric juice as abscissae, a curve was plotted. In this way twelve curves relating peptic activity to the colour produced were made, two for juice obtained after the injection of histamine in known hypersecretory subjects and the remainder for fresh resting and post-prandial juice. The percentage concentration of gastric juice, corresponding to an ordinate value of 0·51 optical density, was then found from each curve and this percentage concentration was taken as equivalent to 50 pepsin units. The figures for percentage concentration of gastric juice were then converted into values in pepsin units/ml. for each curve. Thus if a concentration of 32% contained 50 pepsin units/ml. all the abscissal percentage concentrations for that particular curve were multiplied by $\frac{50}{32}$ to give values in pepsin units/ml. From the 12 recalculated curves a composite curve, based on 138 determinations, was plotted. The values of Table 1 are taken from the curve so produced.

The experimental points shown in Fig. 1 have been selected to include those furthest from the composite mean curve whilst all the determinations made over the range 25–60 pepsin units are shown in Fig. 2.

The method may be used in any laboratory by determining the colour given by the standard reagents with 2 ml. phenol solution (5·0 g./100 ml.). If this optical density be C, then before reading the peptic activity from the curve, all colorimeter readings must be corrected by a factor $C/0{\cdot}51$, since the graph is plotted for our equivalent of the standard, viz. 0·51 optical density.

One of the disadvantages of using gastric juice as a source of proteolytic enzyme, for establishing the relationship between colour produced and peptic activity, is that gastric juice of very high activity is difficult to obtain. With a solution of pepsin, concentrations greater than those found in gastric juice can be used to produce a curve whose range embraces all peptic activities likely to be encountered in gastric juices. In determining the relationship shown in Table 1, the experiments for values of above 150 pepsin units/ml. was made by increasing the digestion time. The validity of this step is supported by the fact that, provided the product of digestion time and dilution is constant, the colour produced is constant. No samples of gastric juice have been encountered with an activity falling outside the range of Table 1.

EXPERIMENTAL BASIS OF METHOD

The details of the preceding method are based upon a series of experiments and the following is a summary of their more important results. The gastric juice in the experiments described was obtained from pooled filtered fractional-test-meal samples adjusted to pH 2·1.

Concentration of substrate. Table 2 shows the effect of variation of concentration of the substrate on the values obtained for peptic activity for a single specimen of gastric juice. A concentration of 5·6 g./100 ml. has been chosen as being the highest concentration conveniently prepared from fresh citrated plasma which can be used in place of the dried product. If untreated plasma is used a different curve relating activity to colour is needed. A high concentration of plasma is desirable to ensure maximal buffering and to increase the range of peptic activity over which the method can be employed (see Beazell *et al.* 1938). If it is desired to husband the substrate, the method has been found to be quite satisfactory for juices of activity

Table 2. *Effect of variations of substrate concentration*

(Optical density ($E \times 100$) corresponding to peptic activity at different concentrations of substrate.)

		Conc. of substrate (g./100 ml.)				
		4	5	5·6	6	7
Activity of dilutions of a gastric juice in pepsin units	9·0	13·4	13·6	13·3	13·0	8·9
	31·5	33·0	35·5	37·8	35·3	35·5
	45·0	44·0	48·0	48·0	48·0	44·3
	63·0	52·0	59·5	57·5	59·5	55·9

Table 3. *Relation between the* pH *of substrate and enzyme activity*

pH	1·4	1·6	1·8	1·9	2·0	2·1	2·2	2·3	2·6	3·5
Optical density ($E \times 100$) corresponding to peptic activity with 3% substrate. Gastric juice ‘P’	22·0	26·0	31·0	34·5	36·0	37·0	37·0	34·0	31·0	18·5
Optical density ($E \times 100$) corresponding to peptic activity with 5·6% substrate. Gastric juice ‘Q’	—	—	—	48·5	51·0	51·2	51·0	48·8	—	—

up to 100 units/ml., with concentrations of 2·4 g. substrate/100 ml. and 4 g./100 ml. trichloroacetic acid. A new curve relating peptic activity to the colour produced is required for each such variation in substrate.

pH *of substrate.* Table 3 shows the relation between the pH of the substrate and rate of digestion for two specimens of gastric juice. The optimum pH lies between 2·0 and 2·2 and it can also be seen that this does not change with the concentration of the substrate.

Influence of variations of pH of gastric juice. Table 4 shows the effect of variations of the pH of a single sample of the gastric juice upon the results for the estimation of its peptic activity. From a ‘pooled’ gastric juice of pH 4·1 three dilutions were made to contain equal concentrations of the original juice at pH of 1·6, 2·1 and 4·1. These diluted samples were then estimated by the routine method, with a substrate containing 3 g. plasma proteins/100 ml. A similar result was obtained with all these specimens. Further the pH of the substrate after the addition of the gastric juices was determined. It may be seen that this pH was not shifted outside the limits for the maximal rate of digestion. These experiments are of great practical importance because they show that it is not necessary to adjust the pH of the gastric juice to that of the substrate even when the latter is used in a dilute solution.

Table 4. *Effect of variations of* pH *of gastric juice on the estimation of its activity*

pH of gastric juice	1·6	2·1	4·1
Optical density ($E \times 100$) corresponding to peptic activity	8·7	8·6	8·8
pH of substrate (3 g./100 ml.) after adding diluted gastric juice	2·05	2·10	2·12

Physiological variables. It has been found that additions of chloride, bile or mucus do not influence the estimation of the activity of gastric juice.

Reproducibility of substrate. To test the reproducibility of the substrate, samples of protein were obtained from various sources (e.g. America and Britain) and used in the estimation of the activity of three samples of gastric juice. Table 5 shows that, despite the difference of origin of the materials employed, the results obtained were surprisingly consistent. Sample CP199/330, which gave a high result, had been shipped to Italy and back where conditions of storage may not have been favourable.

Stability of the substrate solution. The results of experiments made to test the stability of the substrate solution are shown in Table 6. Substrate solutions were made on the

Table 5. *The estimation of the activity of samples of gastric juice with proteins from various sources*

		Optical density ($E \times 100$) corresponding to peptic activity	
Protein substrate	Gastric juice	Individual values	Means
2 May			
Slough serum protein$_1$	A	31·5	
		31·0	31·3
Slough serum protein$_2$	A	28·4	
		29·4	28·9
British plasma protein	A	30·0	
		29·4	29·7
American plasma protein	A	30·2	
		29·4	29·8
11 July			
Plasma proteins LP119/6	B	39·2	
		39·2	
		39·8	
		39·8	39·5
Plasma proteins LP119/122	B	41·2	
		40·0	
		40·0	
		38·5	39·9
Plasma proteins LP119/4	B	39·0	
		40·0	
		39·0	
		40·0	39·5
Plasma proteins LP119/228	B	39·0	
		37·7	
		40·0	
		41·0	39·4
Plasma proteins CP199/330	B	41·8	
		41·0	
		42·0	
		41·5	41·6
Plasma proteins LP119/50	B	38·5	
		39·5	
		40·1	
		39·3	39·4
26 Sept.			
Plasma LP119/50	C	69·5	
		70·0	
		69·0	
		70·0	69·6
Serum 1050–46	C	69·0	
		69·0	
		70·0	
		69·0	69·3

dates shown and kept under the conditions shown. They were later concurrently used to estimate the peptic activity of a gastric juice. It is apparent that the substrate is stable for at least 24 hr. at 20° and for 48 hr. at 6°. The gastric juice has been found to be stable for approximately 6 hr. at 20° after dilution.

Table 6. *Tests of the stability of substrate*

Substrate solution	Date of test	Optical density (E × 100) corresponding to peptic activity: Individual values	Mean
Made up immediately before test. 23 Mar.	23 Mar.	58·9	59·2
		59·5	
Made up 22 Mar. Kept at 20° for 24 hr.	23 Mar.	59·9	60·1
		60·3	
Made up 21 Mar. Kept 4 hr. at 20° followed by 48 hr. at 6° in refrigerator	23 Mar.	60·5	60·4
		60·3	

Trichloroacetic acid. The strength of the trichloroacetic acid has been chosen by trial and error to give a clear filtrate. It should be standardized by titration to 0·350N.

The filtration. A very small amount of the substance producing blue colour is retained by the filter paper, but this is of little consequence as the error is minimized by the use of the curve relating activity to colour. The filtrate is not uniform, and therefore filtration should be allowed to proceed almost to completion as it does in the 15 min. allotted, and the filtrate must be mixed before the 2·0 ml. are pipetted out for analysis. The colour-producing property of the filtrate is only constant up to 30 min. after the beginning of filtration.

The colour-developing reaction. This is influenced by hydrogen ion concentration and by the amount of Folin & Ciocalteu's reagent used. If less alkali than the standard 20·0 ml. 0·25N-NaOH is used the colour develops more slowly to a higher intensity, while with more than 20 ml. the colour develops more quickly but begins to fade sooner. Thus 20 ml. ensures a useful balance between the rate of development and the fading of the colour, which is maintained at a constant intensity for 25 min. under the standard conditions at pH 12·5. The rate of development and persistence of the colour is not influenced by the concentration of the filtrate.

If the colour produced is too dark to be read in the colorimeter, it can be diluted with a convenient volume of water before reading. The true colour can then be calculated.

1·0 ml. of undiluted Folin & Ciocalteu's reagent has been found to be convenient and, further, it appears that when this amount is used errors in measuring produce minimal changes in the final colour developed. The reagent should be admitted uniformly for all tests.

To test the reproducibility of the phenol reagent four samples were collected from various laboratories, where they were kept in refrigerators, and tested against the phenol standard and the Folin & Ciocalteu reagent in use for this work. In spite of the different degrees of yellowness of the samples they gave consistent results with the phenol standard (Table 7).

Table 7. *Test of the reproducibility of Folin & Ciocalteu's phenol reagent from different sources*

(2·0 ml. standard phenol (5·0 ml./100 ml.) were used in each test.)

Source of sample	Optical density (E × 100): Phenol reagent and NaOH	Phenol reagent, NaOH and standard phenol	Difference corresponding to phenol standard
British Drug Houses Ltd. 646256/460909	2·0	52·5	50·5
	2·0	52·5	50·5
Unknown	4·5	55·0	50·5
	4·6	55·0	50·4
Hopkins and Williams Ltd. 1 Nov. 1946	2·0	53·0	51·0
	2·0	53·0	51·0
British Drug Houses Ltd. 650319/470219	2·0	53·0	51·0
	2·0	53·0	51·0
British Drug Houses Ltd. 643791/460424	2·0	53·0	51·0
	2·0	53·0	51·0

Experiments have shown that it is essential to add the phenol reagent with the minimum of delay to obtain consistent results. The consistency obtainable by these procedures was tested by estimating ten samples from a pooled filtrate. The readings of these ten estimations fell between 22·8 and 23·0. It is particularly important that the 50 ml. flask used should be free from contamination with any colour-producing agent. As commercial ethanol contains such substances, it is advised that the final washing of these flasks should be with water.

Differences between duplicates may be due to such contamination, and this can be checked by analysis of another sample of the same filtrate. Substances which reduce the amount of colour produced have not been detected, therefore, if the duplicates differ, the lower reading is probably better.

In the original method of Folin & Ciocalteu (1927), Na_2CO_3 was used as the alkali in the final system. Anson & Mirsky (1932) used NaOH in place of Na_2CO_3 and this has been found the more reliable agent.

The accuracy of the method. Fourteen measurements of the activity of a single sample of gastric juice with one substrate gave a mean reading of 44·0 pepsin units, with a coefficient of variation for individual readings of ±2·64 %. The coefficient of variation of an individual reading in pepsin units for twenty-eight determinations with seven samples of substrate shown in Table 5 for the experiments of 11 July and 26 September is ±2·5 %. Results for sample CP199/330 have been omitted (cf. p. 107).

DISCUSSION

The main principles of the method of Anson & Mirsky (1932) have been followed, but several significant details have been altered. They used tyrosine as a standard, but the appropriateness of this substance is perhaps doubtful since the digestive products giving a blue colour are probably a mixture of tyrosine and tryptophan. What is needed is a stable substance which can be readily obtained in a pure state, and which will produce a consistent colour with the Folin & Ciocalteu reagent. Phenol is such a substance and it has been used to provide the colour standard in this work. Thus the preparation of a pure sample of tyrosine is avoided.

Since the relationship between concentration of phenol and the colour produced in the reaction with the phenol reagent is not linear over a wide range, peptic activity has been related to a standard phenol at one point only. A small divergence from true proportionality is also found for the mixture of tyrosine and tryptophan estimated in the digestion products. Herbert (1946) has confirmed this point for the estimation of phenol with Folin & Ciocalteu's phenol reagent when $NaCO_3$ is used as the alkaline medium for colour development.

The method has proved quite practicable with ordinary citrated plasma so that, in the absence of a supply of dried plasma or serum, it could be used with citrated or whipped ox blood. However, it is unlikely that the supply of plasma or serum will fail, since occasional batches will always be rejected as unfit for transfusion because they are icterogenic. A new relationship between peptic activity and colour would, however, have to be established for fresh plasma. Any alteration of the concentration of the substrate beyond the range 5–6 g./100 ml. would also require a simultaneous modification of the strength of the trichloroacetic acid to ensure that the optimal pH was reached in the colour development reaction.

The method described estimates the total proteolytic activity of gastric juice at pH 2·1 with one special substrate. The practice is to attribute all this activity to pepsin, and the method has therefore been described as a method for measuring pepsin. However, the work of Northrop (1939) suggests that it may later prove necessary to apportion the activity of gastric juice to pepsin, gelatinase and possibly other enzymes. The uncertainty surrounding the source of the activity of the gastric juice seemed in part to justify the completely arbitrary measure of proteolytic activity used in this work.

SUMMARY

1. A method is described for measuring the proteolytic activity of gastric juice with a substrate of dried plasma or serum protein which has been found to be reproducible and convenient.

2. The method allows of the comparison of peptic activity in different laboratories without reference to a standard pepsin.

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The Persistence in the Blood Stream of some Compounds Related to Suramin

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Suramin (Antrypol, Germanin, Bayer 205, Moranyl, F. 309, Belganyl, Naganol, see formula I) displays an extraordinarily marked persistence in the blood stream of man and experimental animals. This property was recognized soon after suramin was first discovered, first, from the long duration of its prophylactic action (Mayer & Zeiss, 1920; Levaditi & Klarenbeek, 1927); secondly, from measurements of the trypanocidal activity of serum and urine of treated animals (Mayer, 1922; Mayer & Menk, 1922). The development of adequate chemical methods of determining it (Steppuhn & Utkin-Ljubovzov, 1924; Lang, 1931; Dangerfield, Gaunt & Wormall, 1938*a*; Boursnell, Dangerfield & Wormall, 1939; Vierthaler & Boselli, 1939), and their use in studying the persistence quantitatively (Lang, 1931; Dangerfield, Gaunt & Wormall, 1938*b*; Boursnell, *et al.* 1939; Vierthaler & Boselli, 1939; Hawking, 1940*a*, *b*) showed that in man, dogs and rabbits, it may persist in the blood stream for as long as 3 months. The form of the blood concentration-time curve is asymptotic (Lang, 1931); during the first few hours after intravenous administration the concentration falls rapidly, but subsequently more and more slowly, until it may be regarded as almost constant over periods of a few days.

Some work has been done on the biochemical and physicochemical characteristics of suramin responsible for this remarkable persistence. No depot of suramin appears to be formed in any tissue. In general, the tissue concentrations are of the same order as, or lower than, the plasma concentration (Zeiss & Utkina-Ljubovtzova, 1930; Boursnell & Wormall, 1939). It must therefore be assumed that the kidney is able to excrete suramin only very slowly, although it can be detected in the urine during the first few days after dosing (Boursnell *et al.* 1939). The most widely held theory advanced to account for this slow excretion is that suramin is bound to protein, either in plasma or cells. Mandel & Steudel (1926) suggested that it was combined in the body with basic proteins of the histone type. They demonstrated that such combination could occur *in vitro*. Collier (1926) showed that suramin in 1% (w/v) concentration could protect horse serum from heat coagulation, and that it combined with globulins (Collier, 1926, 1927). Brunelli (1934) showed that it combined with the euglobulin fraction of horse serum and, to a less extent, with the pseudoglobulin fraction. Boursnell & Wormall (1939) considered that proteins other than the globulins might also be involved; and Dewey & Wormall (1946) demonstrated the combination *in vitro* of suramin with proteins of various types. They precipitated the protein with alcohol in the presence of suramin, and then extracted the protein-suramin complex with ethanol in a Soxhlet apparatus. Large amounts of suramin remained bound to the protein, even after this treatment. It seems likely that the persistence is largely due to this firm combination with the body proteins; however, other factors may be involved. Many drugs that are largely bound to protein are rapidly eliminated, their slow filtration through the glomeruli being augmented by participation of the tubular epithelium in the renal excretory processes. Examples are sulphathiazole, *p*-aminobenzoic acid (Lundquist, 1945) and penicillin (Beyer, Peters, Woodward & Verwey, 1944; Rantz, Kirby & Randall, 1944; Beyer, 1947). Conversely, the most persistent sulphonamides are those that are extensively reabsorbed from the glomerular filtrate by the tubular epithelium (Fisher, Troast, Waterhouse & Shannon, 1943; Earle, 1944). Usually they are also bound to plasma protein. Tubular processes may possibly play a similar part in the persistence of suramin. The strength of the bond uniting it to protein may also be of importance (Gregerson & Rawson, 1943; Rawson, 1943). A further possibility, which does not seem to have received attention, is that suramin exists in solution as colloidal aggregates of sufficiently high nominal molecular weight to hinder passage through the glomerulus. Some support is provided by the known inability of suramin to pass through a collodion membrane (Boursnell *et al.* 1939).

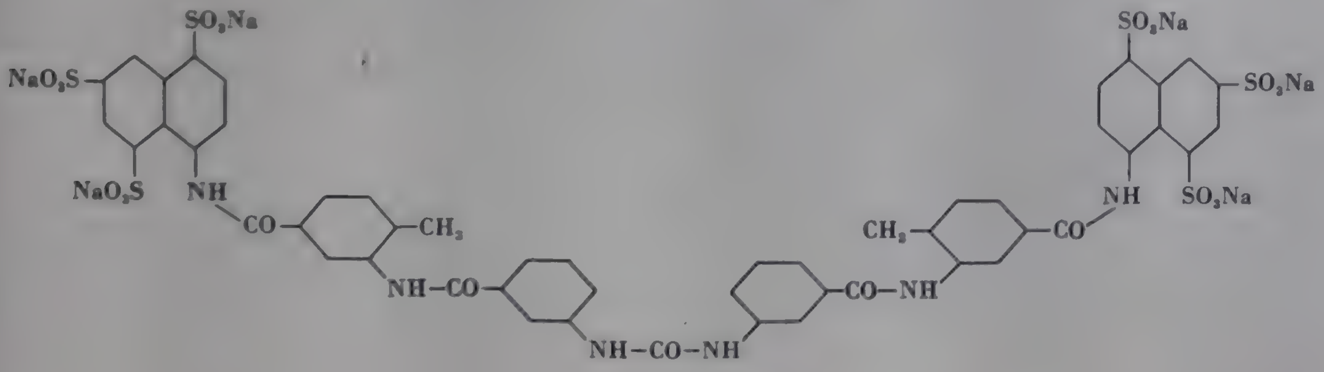
The high persistence of suramin raises interesting questions regarding its degradation by the body. No drug so far adequately examined has proved to be completely unaffected by metabolic processes. If suramin is degraded it must be assumed, either that the process involved is extremely slow, or that the products are themselves highly persistent. The prophylactic action of suramin is associated with the maintenance of a definite blood concentration of about 1·3 mg./100 ml., as determined by the Wormall method (Vierthaler & Boselli, 1939). It is possible that part of this concentration may be contributed by degradation products. The most obvious method of degradation of suramin, a polyamide related in structure to the natural polypeptides, is by hydrolysis to the three constituent aromatic amino-acid residues. The possibility that these are formed *in vivo* was considered by Dewey & Wormall (1946) who showed that a mixture of them, prepared by acid hydrolysis of suramin, was rapidly eliminated. If the postulated degradation gives the constituent amino-acids, it must proceed very slowly. The further possibility that degradation might stop at an intervening stage, yielding a persistent urea or amide, was not examined.

It seemed to the author that an examination of the persistence of compounds structurally related to suramin might provide information on three main problems of interest. First, it might indicate the structural features of the suramin molecule that confer high persistence. Such information would be of general value in the synthesis of prophylactic drugs. Secondly, it might show whether the structural features conferring high persistence are also those conferring high activity. If this were so it could be tentatively assumed that the activity of suramin involves union with an enzyme of the parasite, qualitatively similar to its union with the plasma proteins. Thirdly, if potential hydrolysis products of suramin were examined, their persistence would indicate whether they were likely to be formed *in vivo*.

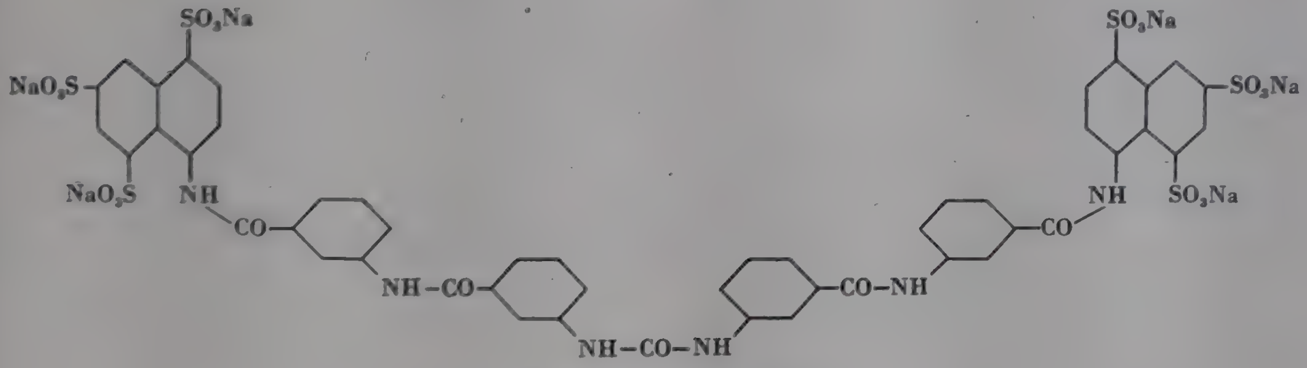
EXPERIMENTAL

Twelve compounds in all were examined. Their structural formulae are shown below. Since they are highly complex, they are referred to throughout by code numbers. Most of them were prepared in these laboratories by Dr R. A. Wilkinson, to whom the author is very grateful.

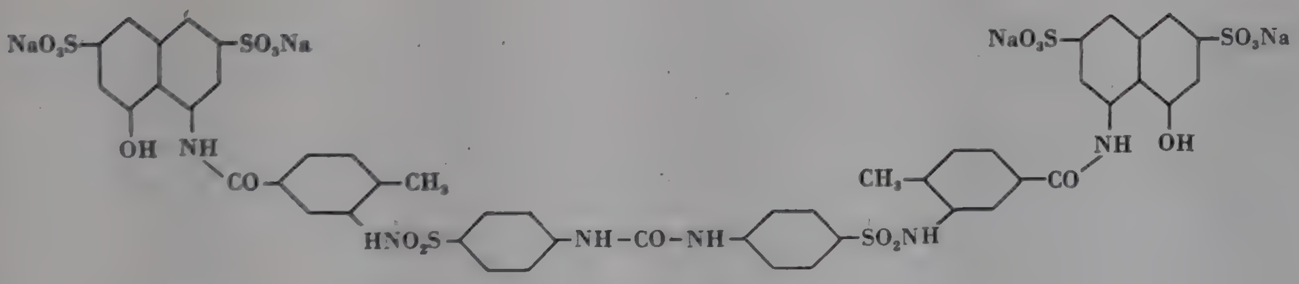
Three methods were used for determining the compounds, based on the standard techniques of diazotization and coupling used in these laboratories (Rose & Bevan, 1944; Rose & Spinks, 1946; Spinks & Tottey, 1946). They represent the first sets of conditions tried that gave adequate (± 10 %) recoveries, and are probably not the best that could be devised. When hydrolysis was necessary, alkaline conditions were used, because the final colour given by normal plasma was thereby reduced. Since some of the compounds were rapidly eliminated, this advantage outweighed those of acid hydrolysis described by Dangerfield *et al.* (1938*a*). It should be particularly noted that no attempt was made to


SO$_3$Na
NaO$_3$S
NaO$_3$S
NH
CO
CH$_3$
NH–CO
NH–CO–NH
CO–NH
CH$_3$
CO
NH
SO$_3$Na
SO$_3$Na
SO$_3$Na

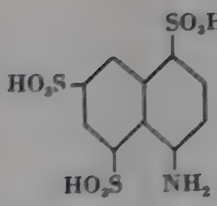
I (suramin)


SO$_3$Na
NaO$_3$S
NaO$_3$S
NH
CO
NH–CO
NH–CO–NH
CO–NH
CO
NH
SO$_3$Na
SO$_3$Na
SO$_3$Na

II (‘demethylated’ suramin)

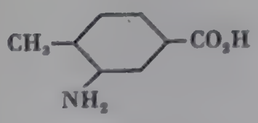

NaO$_3$S
SO$_3$Na
OH
NH
CO
CH$_3$
HNO$_2$S
NH–CO–NH
SO$_2$NH
CH$_3$
CO
NH
OH
NaO$_3$S
SO$_3$Na

III

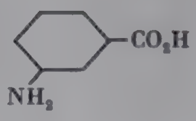

SO$_3$H
HO$_3$S
HO$_3$S
NH$_2$

(as a mixed disodium salt)

IV

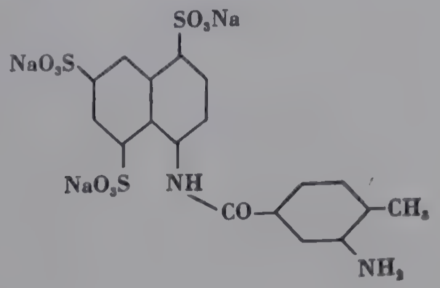

CH$_3$
CO$_2$H
NH$_2$

V

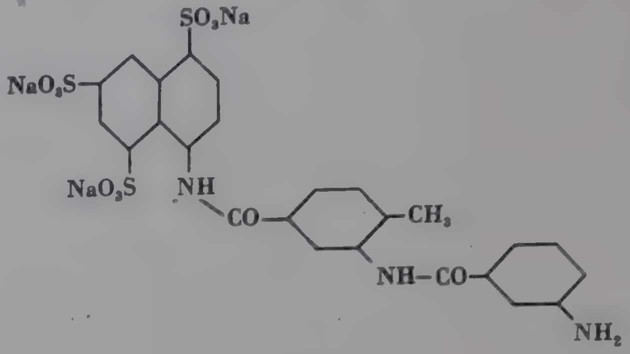


VI

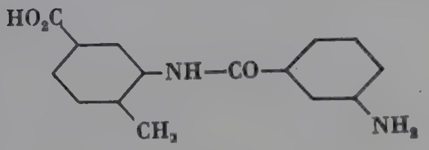
discover the shortest possible time of hydrolysis; 6 hr. was regarded as ample time and used in all experiments.



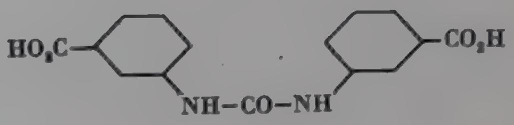
VII



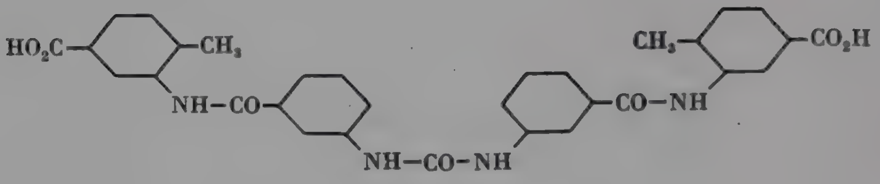
VIII



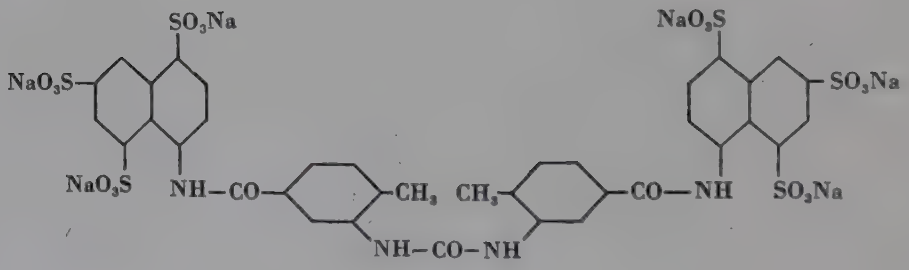
IX



X



XI



XII

Method A, used for compounds V, VI, VII, VIII and IX

Plasma (0·2 ml.) was pipetted into 2 ml. of water in a tube graduated at 10 ml., and treated with 2 ml. of 15% (w/v) trichloroacetic acid. The tube was shaken, allowed to stand for 5 min. and centrifuged at 2000 r.p.m. for 10 min., 0·5 ml. of 0·1% (w/v) $NaNO_2$ was then added; the solutions were well mixed, and adhering protein was removed from the walls of the tube with a thin rod. The tube was then recentrifuged for 5 min. Exactly 20 min. after adding nitrite, 0·5 ml. of 1% (w/v) *N*-β-sulphatoethyl-*m*-toluidine and 2 ml. of 30% (w/v) sodium acetate.$3H_2O$ were added, and the solution was well mixed without disturbing the protein clot. After 20 min. 0·5 ml. of conc. HCl was added, and the volume was made up to the mark with distilled water. The solution was again stirred and the tube was recentrifuged for 5 min. The colour was then read in a photoelectric colorimeter against a series of standards similarly prepared from 0·2 ml. volumes of aqueous solutions containing 0–50 mg. of the appropriate compound/100 ml.

Notes. Coupling of each diazonium compound was approximately complete after 20 min. under the conditions described. A 1 cm. cell and a blue-green filter were used in the colorimeter.

Method B, used for compound IV

Method B was identical with method A except that coupling was allowed to proceed for 40 min. after adding acetate. Even then it was less than 50% complete. The slow coupling of diazotized 1-naphthylamine-4:6:8-trisulphonic acid (IV) is probably due to steric hindrance by the bulky group in the 8-position. The colours produced were very weak. A 2 cm. cell and a green filter were used in the colorimeter.

Method C, used for compounds I, II, III, X, XI and XII

Plasma (1 ml.) was treated with 2 ml. of 2N-NaOH in a tube graduated at 10 ml.; the tube was stoppered with a vaselined glass stopper, and heated at 100° for 6 hr. After cooling, 1 ml. of conc. HCl was added, and the volume was made up to 10 ml. with distilled water; 2 g. of kaolin (B.P.) were then added, and the tube was well shaken for 1 min. The contents were filtered, and 2 ml. of the almost colourless filtrate were treated with 0·5 ml. of 0·1% (w/v) $NaNO_2$ in a tube graduated at 10 ml. After 20 min. 0·5 ml. of 1% (w/v) *N*-β-sulphatoethyl-*m*-toluidine and 1 ml. of 30% (w/v) sodium acetate ($3H_2O$) were added. Coupling was allowed to proceed for 20 min., and 0·5 ml. of conc. HCl was then added. The volume was made up to 10 ml. with distilled water, and the sample was read in the colorimeter against standards similarly prepared from 1 ml. volumes of aqueous solutions of the appropriate compound, ranging in strength from 0 to 50 mg./100 ml.

Notes. Alkaline hydrolysis gave lower blanks from normal plasma than acid hydrolysis. No kaolin was added to the standards. A 1 cm. cell and a blue-green filter were used in the colorimeter.

Recoveries of compounds from plasma. Recoveries of known amounts of the compounds from plasma are shown in Table 1.

Table 1. *Recoveries of known amounts of compounds from plasma*

Compound*	Added (mg./100 ml.)	Found (mg./100 ml.)	Recovery (%)
I	5·0	5·5	110
	10·0	8·5	85
	20·0	22·0	110
	30·0	32·5	108
	50·0	46·5	93
II	10·0	10·4	104
	15·0	14·0	93
	20·0	21·7	108
	30·0	26·9	90
	40·0	38·5	96
	50·0	51·4	103
III	5·0	4·7	94
	10·0	9·1	91
	15·0	13·3	89
	20·0	18·3	91
	25·0	22·7	91
	30·0	23·9	80
	50·0	49·7	99
IV	5·0	5·5	110
	5·0	5·0	100
	15·0	13·5	90
	15·0	16·0	107
	25·0	22·5	90
	50·0	47·7	95
V	2·5	2·5	100
	5·0	5·5	110
	7·5	7·7	103
	12·5	12·5	100
	17·5	17·7	101
	25·0	23·8	95
VI	2·5	2·25	90
	2·5	2·5	100
	5·0	5·5	110
	7·5	8·2	109
	12·5	12·0	96
	15·0	15·7	105
	17·5	18·5	106
	17·5	18·5	106
	25·0	28·0	112
	25·0	23·0	92
VII	5·0	5·0	100
	15·0	17·0	113
	25·0	25·0	100
	25·0	26·1	104
	35·0	36·5	104
	50·0	53·5	107
VIII	10·0	15·0	150
	20·0	21·0	105
	30·0	29·5	98
	50·0	49·0	98
	70·0	66·0	94
	100·0	94·0	94
IX	5·0	5·0	100
	5·0	5·5	110
	10·0	10·5	105
	10·0	10·3	103
	15·0	15·0	100
	25·0	25·0	100
	25·0	27·0	108
	35·0	38·7	111
X	5·0	4·9	98
	10·0	9·7	97
	15·0	14·5	97
	20·0	18·4	92
	30·0	27·9	93
	50·0	49·5	99
XI	5·0	4·85	97
	10·0	12·1	121
	15·0	16·1	107
	20·0	20·8	104
	30·0	35·0	117
	40·0	45·2	113
XII	5·0	5·0	100
	10·0	8·6	86
	15·0	12·9	86
	20·0	15·2	76
	30·0	24·1	80
	50·0	43·5	87

* See p. 112.

Biological tests

Each compound was injected into a marginal ear vein of an adult rabbit in a dose of 100 mg./kg., as a 2·5, 5 or 10% solution of the sodium salt. Oxalated blood samples were taken at intervals from the marginal vein of the other ear, or by cardiac puncture, and immediately centrifuged. The compound was then determined in the plasma by the appropriate method. From this preliminary experiment the approximate degree of persistence of the compound was calculated. It was then administered under the same conditions to a group of three rabbits, blood samples being withdrawn at standard intervals indicated by the preliminary experiment. From the plasma concentrations in the three rabbits a mean plasma concentration-time curve was constructed.

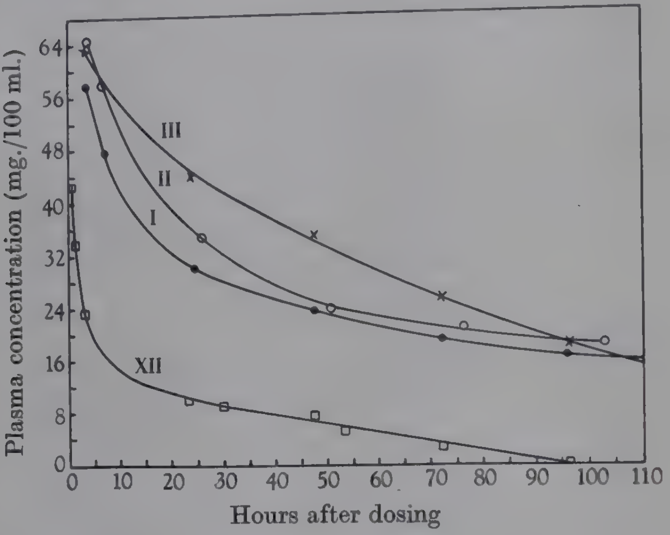


Fig. 1. Mean plasma concentration-time curves of compounds I, II, III and XII following the intravenous administration of 100 mg./kg. to groups of three rabbits.

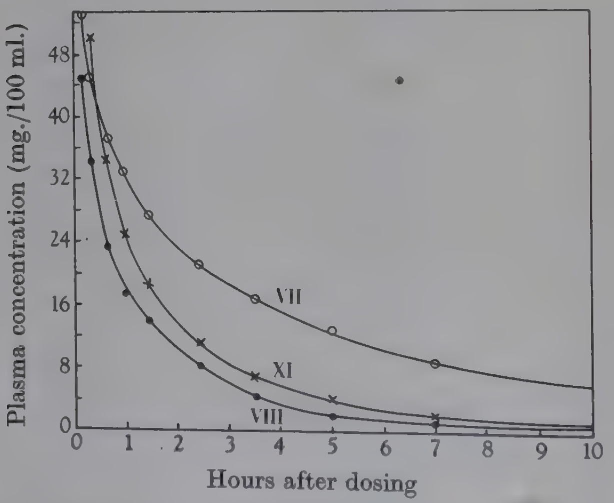


Fig. 2. Mean plasma concentration-time curves of compounds VII, VIII and XI following the intravenous administration of 100 mg./kg. to groups of three rabbits.

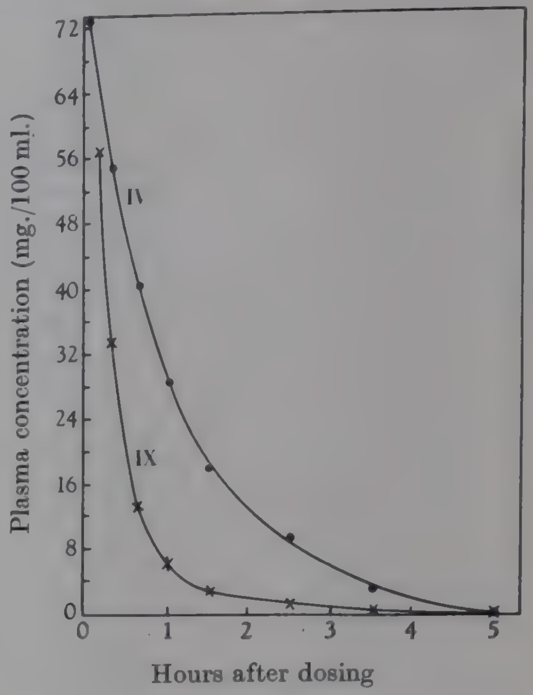


Fig. 3. Mean plasma concentration-time curves of compounds IV and IX following the intravenous administration of 100 mg./kg. to groups of three rabbits.

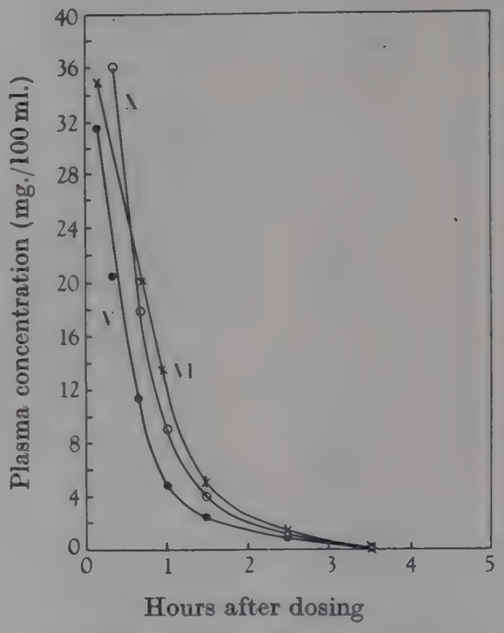


Fig. 4. Mean plasma concentration-time curves of compounds V, VI and X following the intravenous administration of 100 mg./kg. to groups of three rabbits.

RESULTS

The mean plasma concentration-time curves of the twelve compounds are shown in Figs. 1–4. Since the compounds differed widely in persistence the scales of the four curves also differ widely, and should be carefully noted. The compounds are arranged in approximate order of decreasing persistence; this order is: I, II, III; XII; VII, XI, VIII; IV, IX, X, VI, V. The first group, including suramin (I), 'demethylated' suramin (II), and a closely related compound (III), having H acid as an end group instead of naphthylaminetrisulphonic acid, and sulphanilamide as a linking group instead of *m*-aminobenzamide, persists in the blood stream for extended periods. After 5 days all three compounds are still present in the plasma at concentrations of about

16 mg./100 ml. The suramin curve is almost identical with that obtained by Dangerfield *et al.* (1938*b*) under similar conditions. The forms of the other two curves suggest that 'demethylated' suramin is very similar to the parent compound, and that the H acid derivative may be slightly less persistent. Compound XII, an analogue of suramin lacking two aminobenzoic acid links, is intermediate in persistence between the first group and the other, more rapidly eliminated, compounds. It is strongly persistent by ordinary standards, being detectable in the blood stream for 3–4 days. The third group of compounds (VII, VIII and XI) is rather weakly persistent, of about the same order as sulphadiazine (cf. Rose & Spinks, 1946). Each is detectable in the blood stream for 10–24 hr. The remaining five compounds (IV, V, VI, IX and X) are very rapidly eliminated, 1-naphthylamine-4:6:8-trisulphonic acid (IV) somewhat less so than the others. The results on IV, V and VI agree with those of Dewey & Wormall (1946) who examined a mixture of the three obtained by hydrolysis of suramin.

DISCUSSION

The results will be discussed in relation to the three problems outlined in the introduction.

1. *Persistence of suramin in relation to molecular structure*

The results show two main trends. First, compounds containing naphthalenepolysulphonic acids as end groups are more persistent than those containing carboxylic acids as end groups (cf. XI and XII; VII and IX; IV and V). Secondly, persistence tends to increase with increase in molecular weight (cf. XII and I, X and XI). From these two trends, and from individual characteristics of the twelve compounds, the following tentative deductions can be made. First, persistence of the suramin order is displayed only by polyamides containing 6 amino-acid groups (I, II, III as compared with XII). Secondly, high persistence is displayed only by polyamides containing naphthalenepolysulphonic acid groups (I, II, III, XII, VII, as compared with IX, X, XI). Thirdly, the presence of two naphthalenepolysulphonic acid groups appears essential for high persistence (XII as compared with VIII). Fourthly, *m*-amino-*p*-toluic, *m*-aminobenzoic and sulphanilic acid groups may be interchanged in the molecule without marked effect on the persistence (I, II, III). Fifthly, a phenolic group may be introduced without marked effect on the persistence (III). Sixthly, the shape and spacing of the molecule may be much modified without marked effect on the persistence (III).

All these deductions taken together strongly suggest that any symmetrical compound of high molecular weight containing a polysulphonic acid as an end group would show strong persistence. This view is supported by the rather strong persistence of some azo dyes of high molecular weight derived from naphthalenesulphonic acids (cf. Evans Blue, Trypan Blue; Dawson, Evans & Whipple, 1920). The susceptibility of the azo linkage to enzymic reduction is possibly what prevents their being retained in the body as long as suramin.

The fact that the spacing of the molecule does not appear to be of prime importance was not foreseen. It had been tentatively assumed that the persistence was due to the association of both polysulphonic acid groups with basic groups of a protein molecule. The difference in shape between I and III was therefore expected to result in reduced persistence of III. However, it may be that the two compounds combine with different proteins, or at different points of the same protein; and combination of compound with protein through the amide groups might also be possible. In that case, however, the dicarboxylic acid XI would be expected to show higher persistence.

2. *Persistence of suramin in relation to activity*

The low activity of II (Davey, 1943) and the trace activity of III (Browning, 1939), which are both of similar persistence to suramin, show that persistence and activity are due to different structural features of the molecule. The inactivity of many compounds prepared by Fourneau, Trèfouel, Trèfouel & Vallée (1924) which, from these results, would almost certainly be highly persistent, supports this view.

3. *Persistence of suramin in relation to its metabolism*

Compounds IV, V, VI, VII, VIII, IX, X, and XI are the chief potential hydrolysis products of suramin. Each is either very rapidly, or rather rapidly, eliminated. Other possible hydrolysis products, including asymmetrical ureas, would probably behave very similarly (cf. VIII, XI, XII). It can therefore be assumed that suramin either is not hydrolyzed *in vivo* or is hydrolyzed extremely slowly. The latter possibility appears rather unlikely, and the former may therefore be tentatively accepted. Other possible routes of metabolism could, however, be suggested which would probably give compounds of similar persistence to suramin, e.g. the oxidation of the latter to phenols, or phenolic sulphates or glucuronides. The long duration of the prophylactic action of suramin, and the known delicacy of the balance between structure and activity (Fourneau *et al.* 1924) strongly suggest that suramin is relatively resistant to metabolic processes.

SUMMARY

1. The persistence of suramin and eleven related compounds in the blood of rabbits has been investigated following their intravenous administration under standard conditions.

2. Marked persistence is a property of polyamides of high molecular weight that contain naphthylaminepolysulphonic acids as end groups.

3. Carboxylic acids of similar molecular complexity are more rapidly eliminated.

4. There is no relation between persistence and therapeutic activity.

5. Suramin is probably not hydrolyzed *in vivo*.

6. Methods of determining each of the twelve compounds in plasma are described.

The author wishes to thank Miss E. France and Miss R. B. Horrocks for valuable technical assistance.

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The Action of Chymotrypsin and Trypsin on Insulin

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Of the various chemical methods used in attempts to elucidate the structure of proteins, the breakdown by mild hydrolysis seems most promising. The constitution of the resulting fragments which can be isolated may then be ascertained separately. This method has been used to some extent by Gordon, Martin & Synge (1941, 1943) who identified a considerable number of small peptide fragments after partial hydrolysis with hydrochloric acid. Whilst the method undoubtedly throws some light on the order of amino-acids in the protein, a complete structural analysis would be a formidable, if not impossible, task, although it is capable of elucidating the structure of the pentapeptide gramicidin S (Synge, 1944, 1945; Consden, Gordon, Martin & Synge, 1946) and is no doubt capable of considerable extension. A more promising method in the case of a protein is hydrolysis by proteolytic enzymes into larger peptides which can then be further examined.

The essential conditions to be satisfied are: (1) The amount of enzyme added shall not be sufficient to contaminate the digest appreciably. This implies that both enzyme and its substrate must not be contaminated with foreign proteins. (2) No rearrangement of the amino-acids must be brought about by the action of the enzyme. Although synthetic actions of papain have been reported by Behrens & Bergmann (1939), there is at present no evidence that proteolysis brought about by animal proteinases such as pepsin and trypsin is a reversible process. We believe that the isolation of a peptide would provide powerful evidence of its existence as a structural component of the protein.

The work reported here is a preliminary examination of the action of the two trypsins on insulin. Numerous studies of the action of preparations of these enzymes on insulin have been made (for bibliographies see Jensen, 1938; Hill & Howitt, 1936), but much of this work is difficult to interpret, owing to the lack of adequate characterization of the enzyme preparations employed. Moreover, when considerable quantities of impure enzyme have been added, as has been done in some of these experiments, it is impossible to tell whether the split-products are derived from insulin or from foreign proteins accompanying the enzyme.

EXPERIMENTAL

Materials. Trypsin and chymotrypsin were prepared from frozen beef pancreas by the methods of Northrop & Kunitz (1932) and Kunitz & Northrop (1935, 1936), the chief modification being that trypsinogen was converted to trypsin and crystallized by the more recent method of McDonald & Kunitz (1946). The trypsin was only once crystallized, the quantity available being rather small. The chymotrypsin was stored in the form of chymotrypsinogen (eight times recrystallized). When required it was activated by the addition of a very small quantity of trypsin ($<0{\cdot}5\%$) and adjusting the pH to 7·6.

Insulin was a crystalline product prepared by Boots Pure Drug Co. Ltd. A portion was recrystallized three times by dissolving 1 g. in water (170 ml.) containing glacial acetic acid (3 ml.). A solution containing 1 mg. of Zn^{++} in the form of its acetate was added, and the pH was adjusted to 6·6 with ammonia. After dilution to 340 ml., the pH was adjusted to 5·9 with glacial acetic acid, and crystallization took place in less than 1 hr. No significant differences were found between the original and the recrystallized insulin, except that the latter contained a rather higher percentage of non-protein N; the material was not dried by washing with ethanol and ether and therefore contained some mother-liquor.

METHODS

Non-protein N (N.P.N.). This was determined by adding 1 ml. of digest to 0·3N-trichloroacetic acid (5 ml.) at room temperature, allowing to stand for 0·5 hr. and filtering. A micro-Kjeldahl N determination was performed on the filtrate.

Amino N. The sample (1 ml.) was added to 5 ml. of a buffer solution having a pH suitable for arresting enzyme action, i.e. to 0·1N-acetic acid in the trypsin and chymotrypsin experiments and 0·1N-phosphate buffer of pH 7·5 in the case of pepsin. The amino N in a portion of the solution was determined by the Van Slyke method in the usual manner. Sanger (1945) has shown that terminal glycyl residues give an abnormally high value for amino N under the conditions used in the Van Slyke method. Since the terminal residues of the peptides are unknown, no correction has been made, but their characterization largely as glycine residues would introduce a significant error in our estimates of the number of peptide bonds broken.

Total N (T.N.). Three hours' digestion with 1 ml. conc. H_2SO_4 containing about 50 mg. of $CuSO_4$, 50 mg. of K_2SO_4 and a few mg. of Na_2SeO_4 was used. With insulin very little change in the T.N. was observed when the period of digestion varied between 2 and 17 hr.

Free amino-acids. These were determined by the ninhydrin method of Van Slyke, Dillon, MacFadyen & Hamilton (1941). Some difficulty was experienced in getting concordant values, the amount of free amino-acid being so small as to give readings of the same order of magnitude as the blanks.

Diffusion coefficient. A cell of the Anson-Northrop type with a sintered-glass membrane was employed. The cell constant, as determined with 0·1-N KCl, was 1·79 cm.^{2} (mean of three results, two before and one after the actual diffusion experiments). The technique used was similar to that described by Mehl & Schmidt (1937). D_{25} (0·1N-KCl) was taken as 1·46 cm.^{2}/day.

The order of the experiments was as follows. The cell was filled with the digest (18·3 ml.) by suction through the sintered-glass diaphragm. It was suspended in an outer vessel so that the diaphragm just touched the surface of the water (20 ml.) contained therein. The cell was left at the temperature of the experiment for about 24 hr. in order to establish a uniform diffusion gradient in the sintered glass. The water in the outer vessel was then replaced, and after a period of diffusion the concentration of N which had diffused into it was determined. The liquid in the outer vessel was then replaced by 20 ml. of fresh water, and after a further period of diffusion the concentration in the diffusate was again determined. In this way the concentration outside the cell was usually estimated in three successive periods of diffusion. The cell was then opened and the concentration inside estimated. From the analytical results the concentration inside the cell at the beginning of each diffusion period could be ascertained. The diffusion coefficients were calculated by the formula

$$D=\frac{2{\cdot}303\,V''V'}{Kt\,(V''+V')}\times\log_{10}\frac{C_0''}{C_0''-(1+V'/V'')\,C'},$$

where V', V'' are the volumes of liquid outside and inside the cell, C_0'' the initial concentration in the cell at the beginning of each diffusion run, C' the concentration outside at the end, K is the cell constant and t the time. The measured diffusion coefficient was corrected to 20° by the formula

$$D_{20}=D_x\left(\frac{T_{20}}{T_x}\right)\left(\frac{\eta_x}{\eta_{20}}\right),$$

where η_x, η_{20} are the viscosities of water at $x°$ and 20°. The factor is 1·609 at 3°, 1·557 at 4°, 1·511 at 5°, 1·465 at 6°, 1·302 at 10° and 0·870 at 25°. The molecular weight was determined by the formula

$$\log_{10}D_{20} = -4{\cdot}56-(1/3)\log_{10}M,$$

which appeared to give the best fit of recorded values of D_{20} for a variety of proteins and other compounds.

Chymotrypsin digests. The solution was usually made by addition of insulin (0·1 g.) to 1·5 ml. of a phosphate buffer (0·5M, pH 7·6). The pH was then adjusted to about 8·3 by 0·02N-NaOH (3 ml.) and the volume made up to 15 ml. with water. (This higher pH was chosen because the solubility of insulin is too small at a lower pH; insulin is completely stable in this solution and no change in activity was observed in 5 days at 25°.)

Cystine. The digest fractions were hydrolyzed by refluxing with a mixture of equal volumes of 6N-HCl and 90% (w/w) formic acid, usually for 19 hr. The cystine content of the hydrolysate was then determined by the method of Kassell & Brand (1938).

RESULTS

Chymotrypsin

The action of chymotrypsin (Fig. 1) is rapid in the early stages, up to the formation of 50% N.P.N. After that it slows down considerably and a long time is necessary to complete the digestion; in fact, the N.P.N. only reaches values in the neighbourhood of 100% when more chymotrypsin is added. The digestion process thus consists of a first stage, in which a rapid liberation of about half the total nitrogen takes place, leaving the remainder in a form still precipitable by 0·3N-trichloroacetic acid. This is followed by a slow digestion of the remaining peptide bonds. The relation between the liberated N.P.N. and amino N in the earlier stages is linear (Fig. 2), the value of the amino N corresponding to 100% N.P.N. being about 22%. This means that the amount of N.P.N. liberated/peptide bond split is approximately the same throughout the reaction, so far as it has been followed. We can estimate this amount in the following way. In the original insulin the free amino N, as determined by the Van Slyke method, is 4·6% of the total (Chibnall, 1946). Taking the N content of pure insulin as 15·54%, a 'sub-molecule' of molecular weight 12,000 (which is the average minimal molecular weight required by all the analytical results) contains a total of 133 atoms of N, of which 4·6% or 6 atoms are in uncombined amino groups. According to Sanger (1945), two of these belong to lysine and the remaining four are terminal α-groups at the free ends of the peptide chains. In the same way, 22% of amino N in the digest corresponds to 29 amino groups, so that complete digestion with chymotrypsin increases the number of terminal amino groups from 4 to 27. Thus if the breakage of 23 peptide bonds releases 100% of the nitrogen into the 'non-protein' part, assuming linearity as in Fig. 2, each peptide bond broken releases 4·35% of the nitrogen, i.e. 5·8 N atoms.

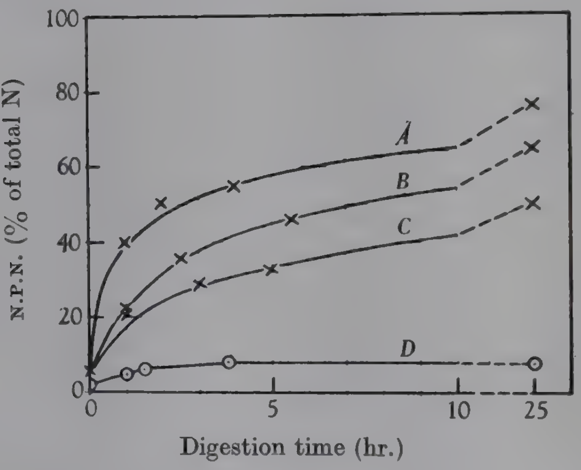

Fig. 1. Action of chymotrypsin and trypsin on insulin. *A*, chymotrypsin, 16 × 10⁻⁴ unit/ml.; *B*, chymotrypsin, 3·2 × 10⁻⁴ unit/ml.; *C*, chymotrypsin, 0·7 × 10⁻⁴ unit/ml.; *D*, trypsin, 4 × 10⁻⁴ unit/ml.

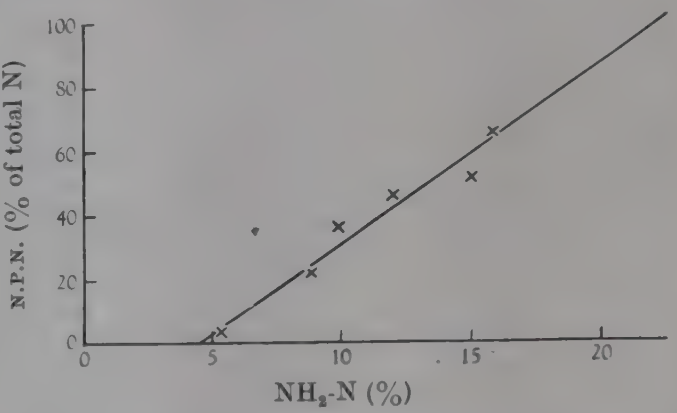

Fig. 2. Action of chymotrypsin on insulin. Relation of amino N to non-protein N.

This estimate was confirmed by a direct determination of the amino N in the trichloroacetic acid filtrate. The solution used was obtained from a digest in which N.P.N. was 58·5%. It was found that the amino N in this filtrate was 22·5%, i.e. c. 1 N atom in 4·5 belongs to a free amino group. After complete hydrolysis with 6N-HCl for 18 hr., the amino N was 79%, i.e. it had increased to 3·5 times its original value, so that the original peptide contains on the average 2·5 peptide bonds for each free amino group, and also 1 atom of N which is not potential amino N. These observations also suggest that the average molecule of N.P.N. is a tetra- or pentapeptide.

Biological activity and splitting. It was found in preliminary physiological tests (see Table 1) that the activity disappeared when about 50% N.P.N. has been formed, i.e. at the end of the rapid reaction, so that the protein-like material precipitated by 0·3N-trichloroacetic acid, which remains at this point, is biologically inactive. An accurate biological assay of the trichloroacetic acid precipitate was kindly made by Boots Pure Drug Co., Ltd. The dried

Table 1. *Effect of chymotrypsin on the physiological action of insulin*

(The amount injected was equivalent to 0·05 mg. insulin. The results given for pure insulin were the average for 6 rabbits, the remainder obtained by injection of a single rabbit.)

Non-protein nitrogen (%)	0	6	28	33	40·3	50	52	55·2
Average decrease in blood sugar 3 hr. after injection of sample (%)	49·2	55	56	54	51	45	0	0

precipitate from a digest with a N.P.N. value of 58·5 % (see p. 121 for particulars) had an activity of 0·68 unit/mg. (with $P=0·95$, limits of error 0·56–0·85). On the assumption that the percentage N in the 'protein-like' material was the same as in insulin, the precipitate contained 55 % of 'protein', so that its activity on a weight basis was 5·4 % of that of insulin. It is probable that the protein-like material at this stage of the digestion contained a little unchanged insulin, amounting to *c.* 2 % of the original insulin.

A further determination of the amount of unchanged insulin remaining at various stages of the digestion was made in the following way. Portions (15 ml.) of a chymotrypsin digest were withdrawn at various times and the 'protein' precipitated by trichloroacetic acid at the usual concentration. At the same times samples were added to equal volumes of 0·1 M-acetate buffer of pH 4·7 giving a final pH of 5·3, close to the isoelectric point at which the solubility of insulin is small. The precipitate formed was filtered off after 1 hr. and the N remaining in the filtrate determined. From these data we were able to find the percentage of the original N present in the trichloroacetic precipitate and the percentage of the original N precipitated at pH 5·3 (Fig. 3). The trichloroacetic acid precipitates were dried, weighed and portions assayed for insulin activity by Boots Pure Drug Co. Ltd.

From the results, the total activity of the trichloroacetic acid precipitate was obtained as a percentage of the insulin activity originally present. It can be seen (Fig. 3) that it agrees quite closely with the percentage of N precipitated at the isoelectric point,* thus providing a fairly convincing proof that (1) the material precipitated at pH 5·3 is, in fact, unchanged insulin and (2) that trichloroacetic acid precipitates, in addition, material which is not insulin and which does not contribute appreciably to the observed residual activity.

Nature of the split-products. It seemed probable that the initial stage of the reaction consisted of a splitting off of small peptides leaving a compact protein-like residue. In order to find what proportion of the liberated nitrogen, if any, was derived from free amino-acids, a determination of these latter formed in the first stage was made by the ninhydrin method. The results obtained are given in Table 2. It is evident from these figures that the free amino-acids formed are only a small percentage (about 2 %) of the whole N.P.N. liberated. In the second example shown, the free amino-acid N formed is 1·23 % of the total N; i.e. it is equivalent to 1·6 N atoms per stoicheiometric unit of 12,000.

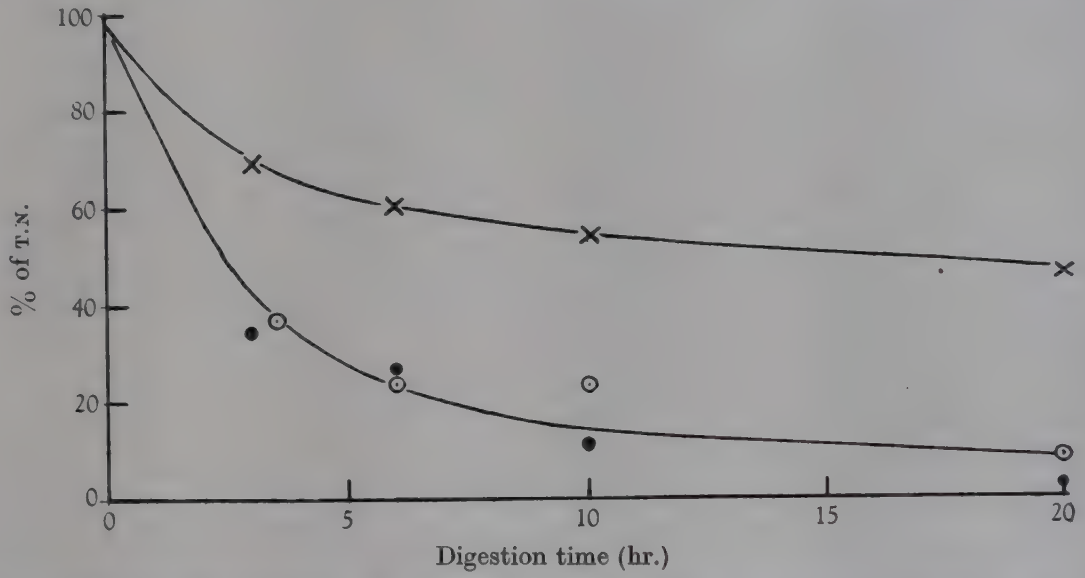

Fig. 3. Action of chymotrypsin on insulin. × Percentage N precipitated by 0·25 N-trichloroacetic acid. ⊙ Percentage N precipitated at the isoelectric point, pH 5·3. ● Percentage of original insulin activity found in trichloroacetic precipitate.

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* The T.N. value of the 10 hr. isoelectric mixture is high because, owing to the time at which this sample was taken, it had to be frozen for a time and filtered about 12 hr. later. After 20 hr. the insulin assay gave a value which was definitely less than that in the isoelectric precipitate, so that in this case the precipitate probably contained material which was not insulin.

Thus between one and two molecules of free amino-acid are formed from the submolecule; i.e. of the 11 peptide bonds broken, one or two may liberate a terminal amino-acid. It is, of course, quite possible that this free amino-acid arises from secondary actions. The greater part of the non-protein fraction must thus consist of peptides with an average chain length, as estimated above, of 4–5 residues.

Table 2. *Products of chymotrypsin digestion*

Digestion period (hr.)	3	30·5
Non-protein nitrogen (% of total N)	31	47·5
Free amino-acid N (% of N.P.N.)	1·8	2·6
Amino N (% of T.N.)	10	12·7
Approx. no. of peptide bonds broken/unit of mol.wt. 12,000	7	11

A further confirmation of this calculation was obtained by determining the molecular weight of the digestion products by the diffusion cell. Insulin (0·5 g.) was digested with chymotrypsin for 17·5 hr. at 25°, the N.P.N. at the end of this period being 58·5%. Trichloroacetic acid was then added to a final concentration of 4%, and after 0·5 hr. the precipitate was filtered off (dry wt. 340 mg.). The filtrate deposited some further precipitate on standing which was removed and the pH was then adjusted to 4·4. This filtrate was then put in the diffusion cell, and after preliminary contact with water to set up the diffusion layer, the amount diffusing out in three successive portions of initially pure water was determined. From these results, and the final concentration in the diffusion cell, the diffusion coefficients were determined and corrected to 20° (Table 3). The values found correspond to a molecular weight of 800±200, which is in good agreement with the figure estimated above. It will be observed that the values of D diminish in successive determinations; this may be due to material of lower molecular weight diffusing out first.

Table 3. *Diffusion coefficient of trichloroacetic acid filtrate*

Temp. (°)	Diffusion time (hr.)	Concentration in 20 ml. diffusate (mg. N/ml.)	$D \times 10^6$	$D_{20} \times 10^6$
5	66·5	0·013	2·05	3·26
5	76·5	0·013	1·87	2·97
4	90	0·013	1·68	2·77

Final conc. in diffusion cell (18·3 ml.) was 0·268 mg. N/ml. Mean D_{20} sec.$^{-1} = 3{\cdot}0 \pm 0{\cdot}3 \times 10^{-6}$.

The trichloroacetic acid precipitate was now dissolved (4 mg./ml.) in 0·02 N-NaOH and the pH adjusted to 8·0. This liquid was put in the diffusion cell and after a preliminary contact with water for 24 hr. the amounts of N diffusing out in successive times were determined (Table 4).

Trichloroacetic acid thus divides the digest into a soluble part, consisting of comparatively small peptides of mol.wt. *c.* 800 and an insoluble part of average mol.wt. *c.* 4000. The former makes up about half the molecule. The insoluble part has a molecular weight which agrees quite well with that of a submolecule of mol.wt. 12,000 from which rather more than half the residues (i.e. about 60) have been removed. The action of chymotrypsin therefore appears to be a removal of the outer parts of the insulin molecule leaving a fairly massive nucleus. This process is complete at the end of the first stage of the digestion (50% N.P.N.). In the later slow stage the massive nucleus is decomposed into smaller non-precipitable fragments.

It is surprising that trichloroacetic acid can precipitate molecules with molecular weight as low as *c.* 5000, well below the order of magnitude usually considered as proteins. This finding must cause some revision in our ideas of what constitutes the 'protein' and 'non-protein' parts of a digest. In view of this we determined the effect of varying the trichloroacetic acid concentration on the amount precipitated, with the following results: 1 ml. digest was added to 5 ml. of trichloroacetic acid of concentration shown:

Conc. of trichloroacetic acid (N)	0·15	0·3	0·6	1·0
Percentage of T.N. remaining as N.P.N.	74	51	48	47

There is thus no significant increase in the proportion of N precipitated at concentrations above that usually employed (0·3 N). When 1 ml. of the digest was added to 9 ml. of acid tungstate, prepared according to Van Slyke & Hawkins (1928), an even greater percentage, viz. 61·6% of T.N., was precipitated.

When added to an acidified chymotrypsin digest, pepsin produces little further splitting. For example, a chymotrypsin digest which contained 14·2% amino N, was acidified to pH 2 and 9×10^{-4} pepsin unit/ml. was added. After 24 hr. the amino N was 15·0%.

Distribution of cystine and of amino N. Since treatment of the 50% digest with trichloroacetic

Table 4. *Diffusion coefficient of trichloroacetic acid precipitate*

	Temp. (°)	Diffusion time (hr.)	Concentration in 20 ml. diffusate (mg. N/ml.)	$D \times 10^6$	$D_{20} \times 10^6$
Series 1	4	65·5	0·007	1·02	1·68
	4	96	0·006	1·24	2·04
Final conc. in diffusion cell, 0·223 mg. N/ml.					
Series 2	3	96	0·0066	0·93	1·58
	3·5	96	0·0063	0·91	1·54
	5	123	0·010	1·18	1·88
Final conc. in diffusion cell, 0·213 mg. N/ml.					

Mean D_{20} sec.$^{-1} = 1{\cdot}74 \pm 0{\cdot}2 \times 10^{-6}$, corresponding to a mol.wt. of about 4000±1500.

acid divides it into two fractions, the distribution of the more important amino-acids between them is of interest. We report below two preliminary experiments on the distribution of cystine. They are not entirely satisfactory, as only about 90 % of the total cystine is accounted for, but this loss can hardly affect the main point which emerges.

Exp. 1. Recrystallized insulin (0·50 g.) in 90 ml. solution, pH 8·2, was incubated with chymotrypsin (3 mg. protein N). After 17·5 hr., N.P.N. was 58·5 %. To 84 ml. of digest was added trichloroacetic acid to 0·24 N; the precipitate, which was filtered off after 30 min., amounted to 340 mg. Both precipitate and filtrate were hydrolyzed with the HCl-formic acid mixture for 19 hr. and cystine contents of the hydrolysates were determined (Table 5).

Table 5. *Distribution of cystine in the soluble and insoluble fractions of a partial digest of insulin*

(The figures of T.N. and cystine N are expressed in mg./10 ml. of original digest.)

	Total nitrogen (mg.)	Cystine N (mg.)	Cystine N × 100 / Total N
Exp. 1:			
Digest	8·05	—	—
Precipitate	3·40	0·504	14·7
Filtrate	4·67	0·114	2·5
Exp. 2:			
Whole digest	7·67	0·618	8·07
Precipitate	4·24	0·476	11·23
Filtrate	3·38	0·151	4·47

In the original solution the N.P.N. was 6 % of the whole, so that the original protein N was 63·5 mg. This should contain 5·95 mg. of cystine N (9·36 %), of which 5·19 mg. (i.e. 87 %) were accounted for.

Exp. 2. In this case the final concentration of trichloroacetic acid was 0·25 N; the precipitate which formed was filtered off but not dried. It was prepared after 33·7 hr. digestion (with lower chymotrypsin concentration) when N.P.N. was 44·1 %. The precipitate was dissolved in a little N-KOH and diluted. Digestion with the HCl/formic acid mixture was carried out for 8 hr. The results are given in Table 5. In this experiment the sum of the cystine concentrations in the two fractions agrees with that determined for the original digest, but both are only 90 % of that expected for the total cystine of the insulin.

In both experiments the proportion of cystine in the precipitate is considerably greater than that in the filtrate, and the ratio is greater for the digest which has been digested further.

The proportion of amino N in the precipitate (55 % of T.N.) was found to be 8·7 %, which means an average chain length in the precipitated material, assuming the same average composition as insulin, of 9–10. This also fits in with the general picture that the enzyme has removed about 10–12 peptide fragments averaging 6 residues from the original submolecule of 106 residues in 4 chains, leaving about 40 residues in 4 chains.

Trypsin

Contrary to statements in the literature, trypsin itself had a very slight action on insulin. When 24×10^{-4} unit of trypsin was added to 5 ml. of a solution containing 17 mg. of insulin/ml. at pH 8·2, the increase of N.P.N. was only 4 % in 24 hr. at 25° (Fig. 1). Insulin was recovered from a solution containing 5·5 mg. insulin and $1{\cdot}4 \times 10^{-4}$ unit of trypsin/ml. after standing at 2·5 hr. at 25° and 16 hr. at 5°. From 14 ml. of this solution which originally contained 77 mg. insulin, 75·4 mg. of insulin were crystallized. An accurate assay of this material was kindly made for us by Boots Pure Drug Co. Ltd., who found that it had 4·03 % moisture and an activity of 22·96 units/mg. corresponding to 23·93 units/mg. of dried crystals. It was therefore indistinguishable from the purest insulin. A much stronger trypsin solution (1 ml.; 0·73 mg. protein N, 800×10^{-4} unit/ml.) was added to 15 ml. of an insulin solution at pH 8·4; this produced about 10 % N.P.N. in 4 hr. and 50 % N.P.N. in 24 hr. Such an action might be produced by the presence of approximately 0·5 % of chymotrypsin in the trypsin. The trypsin was therefore tested by the milk-clotting method as used by Kunitz (1935) and Herriott (1938) standardized with pure chymotrypsin; the activity corresponded to the presence of 0·4 % chymotrypsin. It was therefore concluded that the slight action observed was due to chymotrypsin present and that trypsin itself has no significant action at pH 8·2. In order to find if any action of trypsin occurred at a lower pH, a solution was made containing 1 mg. of insulin/ml. at pH 7·0. Trypsin produced no appreciable change of amino N and no decrease in biological activity.

Trypsin can, however, hydrolyze further the products after digestion of chymotrypsin, if present in considerable amount. A chymotrypsin digest to which trypsin was added at a concentration of 0·05 mg./ml. changed very little in 19 hr.; but at a concentration of 0·5 mg./ml. the amino N rose overnight from 15·8 to 20·8 %, while the increase in the same time without trypsin was only 3 %. It follows that the rate of digestion of insulin by trypsin and chymotrypsin together will be greater than the sum of their separate effects.

SUMMARY

1. The action of crystalline trypsin and chymotrypsin on insulin has been studied. Crystalline trypsin has no appreciable effect on insulin, but is capable of digesting the products formed after chymotrypsin action.

2. The action of chymotrypsin occurs in two stages. The first stage consists of a rapid action leading to the formation of about 50 % non-protein

N as determined by precipitation with 0·25N-trichloroacetic acid. In the second stage, the non-protein N rises slowly to the 100% value. The biological action disappears at about the end of the first stage. Diffusion experiments showed that the 'protein-like' material left here had a molecular weight of only 4000, whilst that of the soluble fraction was about 800. The initial action of chymotrypsin thus involves the cleavage of about 10 or 11 peptides containing on the average 5 or 6 residues from each submolecule, leaving a fairly massive core. The latter is broken down in the second stage of the action.

3. The amino-N content of the 'core' indicates a probable chain length of 10–12 residues. The greater part of the cystine (from 75 to 85%) is in the core.

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The Action of Pepsin on Insulin and the Plastein Question

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The course of the action of pepsin on insulin, and the products of the digestion, are quite different from those observed with chymotrypsin. On the whole, the breakdown produced by pepsin is less extensive than that observed with chymotrypsin. The most interesting feature of the action is the diminution, under certain conditions, of the apparent 'non-protein' nitrogen in the digests. This phenomenon appeared to be similar to the process of plastein formation, which has been known for a considerable time. Wasteneys & Borsook (1924, 1925)* reported that when a peptic digest of egg albumin is concentrated, and then incubated with more pepsin at pH 4 (at which pH the proteolytic action of pepsin is slight), the 'non-protein' nitrogen, determined as the nitrogen soluble in the presence of 2% trichloroacetic acid, is diminished. They regarded this as evidence of the synthesis of protein-like material, 'plastein', by the action of pepsin at pH 4, citing as contributory evidence the fact that the plastein, when filtered off, contained an appreciably smaller proportion of amino N than the original digest. They studied the effect of such variables as pH, temperature and concentration, and reported that when boiled pepsin was added instead of the active enzyme, no synthesis was observed. The matter was re-examined by Folley (1932), who concluded that there was no proof of resynthesis and found that plastein formed from egg albumin had a comparatively low molecular weight.

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* Wasteneys & Borsook give references to earlier observations of this kind.

Similar observations were made with a peptic digest of insulin by Fisher & Scott (1933), who used the decrease of material soluble in 3·3 % trichloroacetic acid as the criterion of plastein formation during incubation at pH 4. Haddock & Thomas (1942) found that trypsin had a similar action at pH 5·7 on the peptic digest of insulin, as had papain on both peptic and papain digests. The circumstances under which insoluble material is formed in insulin digests have been investigated in some detail in this paper.

EXPERIMENTAL

The pepsin was a crystalline product presented by Parke, Davis and Co. It was kept at −10° as a 2% stock solution in 60% glycerol adjusted to pH 5 with sodium acetate and acetic acid; dilutions were prepared as required. The activity as determined by the method of Anson (1938) was 0·54 unit/ml.

Non-protein nitrogen (N.P.N.). At first the digest was added to boiling trichloroacetic acid (0·3 N) in order to inactivate the pepsin, but this was unsatisfactory owing to the uncertain amount of evaporation, and it was found that addition of trichloroacetic acid at the same strength at room temperature gave nearly the same results (Table 1).

The trichloroacetic acid mixture was not allowed to stand too long before filtration, as a slow precipitation of insoluble material might occur. Certain of the pepsin digests which gave no immediate precipitate with trichloroacetic acid gave one after standing for 1–2 hr. The time of standing was standardized at 30 min., which in certain cases of complete digestion gives too low a value for N.P.N.

Table 1. *The determination of non-protein* N (N.P.N.) *in an insulin digest by trichloroacetic acid* (T.C.A.) *at room temperature and at the boiling point respectively*

(Insulin (60 mg.) in 12 ml. of 0·05 N-HCl; 1 ml. of 1/100 dilution of stock pepsin.)

Time after adding pepsin (hr.)	N.P.N. (mg./ml.)	
	T.C.A. at room temp.	T.C.A. (boiling)
1	0·21	0·21
2·5	0·31	0·29
5	0·62	0·53

Peptic digests. The solution used usually contained 5 mg. of insulin dissolved in 1 ml. of 0·05 N-HCl, to which was added the desired amount of pepsin, usually at a dilution of 1 : 100 of the stock solution.

RESULTS

Extent of the action. The N.P.N. rises smoothly and quickly to values close to 100 %, while the amino N reaches and remains at a constant value (with small pepsin concentrations) of about 12–13 % (Fig. 1). This may be followed by a very slow action of pepsin on the primary split-products, the amino N reaching 13–14 % in 24 hr.; with concentrations of pepsin 5 or 10 times as great the amino N reached 16 % in 2 days. The 13 % stage is, however, fairly definite and easily reproduced. During this initial action there is a linear relation between the N.P.N. and amino N (Fig. 2), which indicates that the amount of N.P.N. liberated/peptide bond split is the same throughout the action of the enzyme. Thus the hydrolysis of the peptides initially formed is relatively slow.

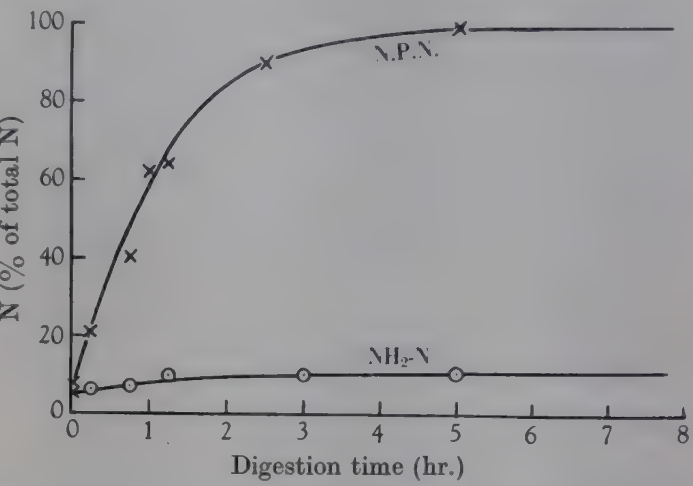

Fig. 1. Action of pepsin on insulin at pH 2. Change of amino N and N.P.N. with time. Pepsin units/ml. of digest, 3×10^{-4}.

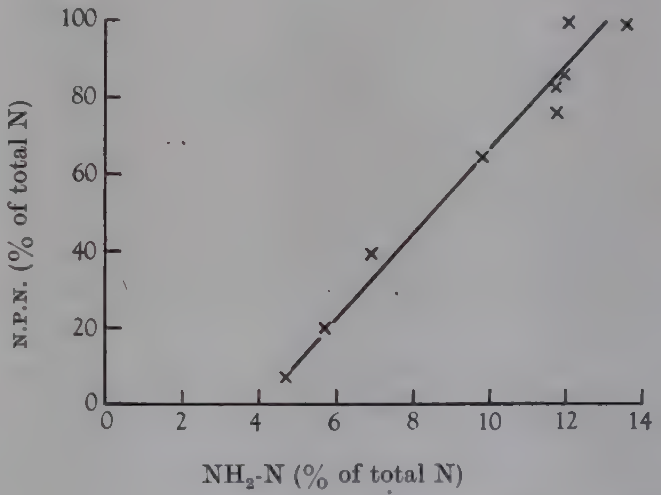

Fig. 2. Action of pepsin on insulin. Relation between amino N and N.P.N.

By the same method of calculation as used in the previous paper (p. 118), it was found that 13 % of amino N in the digest corresponds to 17 amino groups, so that the digestion has increased the number of terminal amino groups from 4 to 15. The average peptide chain in the digest has a molecular weight of 12,000/15 = 800 and thus contains about 7 amino-acid residues.

This gives, of course, no information about the uniformity of the products. The amount of uncombined α-amino-acids in the digest as determined by the Van Slyke ninhydrin method was 0·7 % of the total N. It follows from this that in the digestion product from one unit of 106 residues (mol.wt. 12,000), less than one is present as uncombined amino-acid, i.e. of the ten peptide bonds broken not more than one belongs to a terminal amino-acid. Indeed, the small amount found may be the result of a secondary reaction.

Diffusion coefficient. A peptic digest was prepared by mixing 200 mg. insulin in 40 ml. of 0·05 N-HCl with 3 ml. of a 1 : 200 dilution of the stock pepsin. After standing overnight at 25° it gave no immediate precipitate with trichloroacetic acid. To destroy the pepsin, the digest was now neutralized to pH 7·2 and heated to 80° for 3 min. The precipitate thus formed was filtered off and when dried amounted to 42 mg., i.e. to about 20% of the original insulin. The nature of this 'heat precipitate' is discussed below. Diffusion coefficients were determined on the filtrate (Table 2).

Table 2. *Diffusion coefficient of digest filtrate*

	Temp. (°)	Diffusion time (hr.)	Conc. in 20 ml. diffusate (mg. N/ml.)	$D \times 10^6$	$D_{20} \times 10^6$
Series 1	25	23·5	0·0075	2·21	1·92
Final conc. inside cell, 0·453 mg. N/ml.					
Series 2	10	49	0·009	1·52	2·05
	5	47	0·006	1·07	1·71
	6	48	0·007	1·32	2·02
Final conc. inside cell, 0·359 mg. N/ml.					

Average D_{20} sec.$^{-1} = 1·92 \times 10^{-6}$.

The average molecular weight of a peptide corresponding to the diffusion coefficient (Table 2) is 3000 ± 1000, which is significantly higher than that of the average peptide chain in the digest. It follows that the diffusing molecule must contain on the average about 4 peptide chains. These might be held together in various ways, but the disulphide bonds, of which there are only 1·5 in a peptide of mol.wt. 3000, are insufficient by themselves for this purpose.

The plastein question

In the course of our experiments we noticed on one occasion that the N.P.N., which had been nearly 100% after digestion for 5 hr., apparently diminished to approximately 50% after 24 hr. without any diminution of the amino N. This was found to be due to the fact that in the latter determination the trichloroacetic mixture had stood for an abnormally long time before being filtered. The following experiment shows the nature of this action. A series of tubes was made up containing 1 ml. of a fresh peptic digest and 5 ml. of 0·3 N-trichloroacetic acid and the mixtures were allowed to stand at room temperature. Precipitate was first observed after 1 hr. At various intervals the precipitates in the separate tubes were filtered off and the N.P.N. was determined; the results are given in Table 3.

Table 3. *Decrease of non-protein* N (N.P.N.) *in a peptic digest of insulin after various periods*

Time (hr.)	N.P.N. (mg./ml.)	N.P.N. (% of total N)
1·5	0·57	88·0
3	0·36	55·4
7	0·34	52·5
24	0·29	44·7

The precipitation of some material by trichloroacetic acid can hardly be due to pepsin, since the latter is inactive in such a solution. However, it appeared possible that it was caused by a hydrolytic action of the acid.

It will be recalled (p. 124) that, when a peptic digest is neutralized and heated to 80° for a few minutes, a precipitate is formed which may amount to 20% or more of the original insulin and continues to form in the filtrate at a slower rate at lower temperatures. Indeed, when the filtrate was frozen and stored at −10°, it was usually found that some insoluble matter was present on thawing. It therefore appeared that the peptic digest undergoes some change on being kept at certain acidities, which results in the formation of material which is both insoluble by itself and in the presence of trichloroacetic acid. In order to find under what circumstances it is formed, a peptic digest was made which gave no immediate precipitate with trichloroacetic acid. The pH of measured samples of this digest was adjusted to different values. After standing for various times at 25°, samples were added to 0·3 N-trichloroacetic acid, the precipitate formed after 30 min. was filtered off, and the N in the filtrate determined (Fig. 3). Except in the alkaline solution a considerable drop in N.P.N. occurred. The percentage of amino N in the whole mixture was determined by the Van Slyke method (Table 4). There is a slight

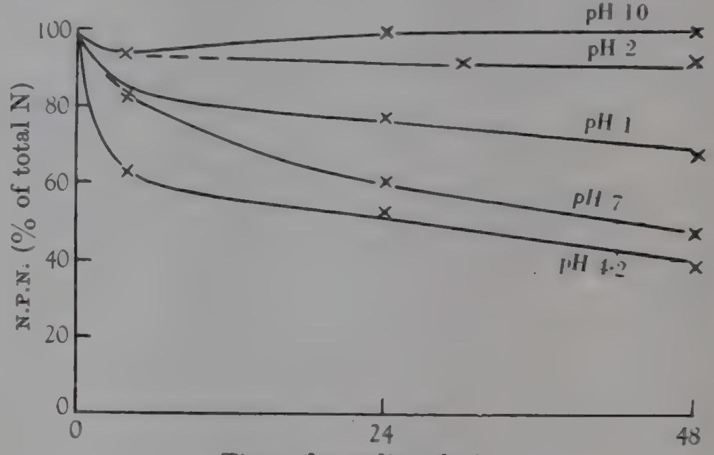

Fig. 3. Changes in peptic digest at 25° with pH.

Table 4. *Change of amino* N *and non-protein* N *with time in peptic digest*

	Original	After standing for 24 hr. at pH 1	4·2	7·0	c. 10
Amino N (% of total N)	13·0	13·0	12·7	13·0	13·7
N.P.N. (% of total N)	100	80·8	54·3	60	100

diminution of the amino N in the three solutions in which N.P.N. has diminished, amounting at most to about 1%. This would correspond to the resynthesis of one peptide bond out of the eleven broken; but it is doubtful whether the determinations are sufficiently accurate, since they are made with a mixture of solid and liquid. It is quite possible that under these circumstances small variations would occur with constant reaction time according to the amount of solid. At any rate, the figures do not support the suggestion that any considerable synthesis of peptide bonds, corresponding to the amount of solid formed, has occurred.

In order to find if the changes observed are due to the pepsin still remaining in the solutions, a further series of experiments was made in which the peptic digest was heated to 60° for 5 min. at pH 7·8 before adjusting the pH to its final value. It was shown that the activity of the pepsin was completely destroyed by this treatment. The pH was then adjusted by the addition of HCl to 2·0, 4·0 and 7·0, and after standing for various times at 25°, samples were taken for analysis. The results are shown in Fig. 4.

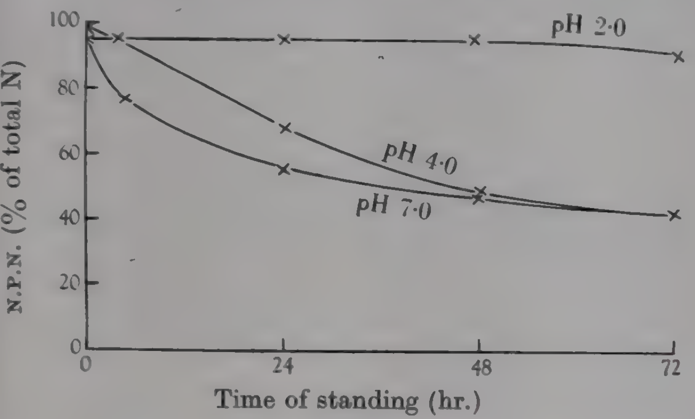

Fig. 4. Changes in peptic digest at 25° with pH after inactivation of the pepsin.

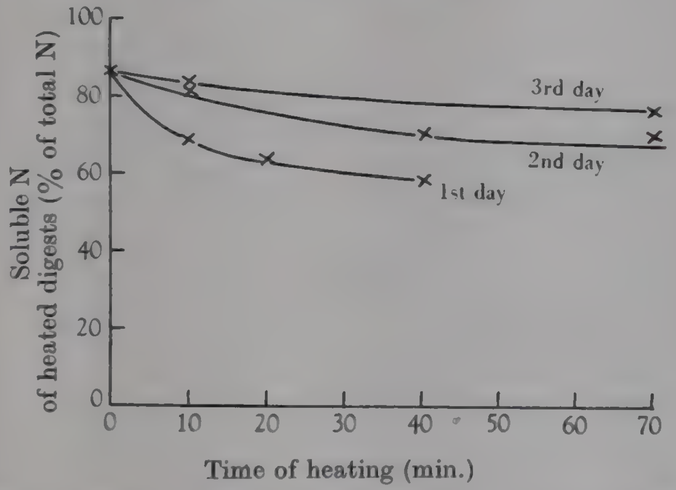

Fig. 5. Effect of age of peptic digest on amount of precipitate formed on heating.

At pH 4·0 and 7·0 the N.P.N. diminishes steadily, reaching about 50% after 2 days, while there is very little change with time when the pH has been restored to its original value. This experiment shows that the presence of active pepsin has nothing to do with the drop of the N.P.N. observed in our experiments.

In the course of this work it was noticed that the quantity of precipitate obtained on heating appeared to be less with an old digest than with a fresh one. To test this a peptic digest was prepared in the usual way. After 18 hr. a portion was neutralized to pH 7 and heated for various times. The precipitates formed were removed by filtration and the amounts of N remaining in the filtrates were determined. The same process was repeated with digests 42 and 66 hr. old (Fig. 5). It can be seen that the amount of precipitate obtained diminishes with the age of the digest.

In this experiment the amount of N precipitated by trichloroacetic acid was also determined (Table 5). In nearly every case the amount agrees with or is only slightly less than the amount precipitated by heating, thus confirming the suggestion that the material precipitated by heating is identical with that precipitated by trichloroacetic acid.

Table 5. *Insoluble matter formed on heating digest*

Age of digest (hr.)	Time of heating (min.)	Percentage of N not precipitated by heat	Percentage of N not precipitated by T.C.A.
18	10	69	69
	20	64	61
	40	59	56
42	10	84	79
	40	71	75
	70	71	65
66	10	85	87
	70	77	74

Properties of the precipitate and filtrate. The precipitate used was obtained from 500 mg. insulin at the usual concentration, digested with 2 ml. of a 1 : 50 dilution of the stock solution of pepsin for 17 hr. The digest was neutralized and heated to 75° for 10 min. The dried precipitate (6A) amounted to 88 mg. After being frozen for 1 week a further 83 mg. of precipitate (6B) was removed. The total precipitate, allowing for filtrate used in tests, was about 40% of the insulin taken.

The precipitate 6A dissolved slowly (1 mg./ml.) in 0·05N-HCl. Its total N amounted to 15·4% of the dry weight. The precipitate is complex. The following amino-acids have been identified in an acid hydrolysate in a preliminary analysis by paper chromatography: aspartic acid, cystine, tyrosine, glutamic acid, glycine, alanine, leucine. There are almost certainly other amino-acids present, as several spots were not identified. The amino N as a percentage of total N in the precipitate 6A, as determined by Van Slyke's method, was 8·2%; in 6B, 8·7%, and in the filtrate from which both 6A and 6B had been removed, 16·2%.

It can be seen that the process of heating divides the digest into two parts containing respectively a smaller and greater proportion of amino N than the original digest. It is impossible to determine the average chain lengths in the fractions without knowing the distribution of the lysine, etc., but on the assumption that the average composition of each is similar to insulin, the precipitates have a peptide chain length of about 10–12 residues, while the filtrate has an average chain length of about 6. The molecular weight of precipitate 6A was determined in the diffusion cell in the usual way (Table 6).

Table 6. *Diffusion coefficient of heat precipitate*

Temp. (°)	Diffusion time (hr.)	Conc. in 20 ml. diffusate (mg. N/ml.)	$D \times 10^6$
25	41	0·0025	1·35
25	72	0·005	1·58
25	72	0·004	1·32

Original conc. in cell, 0·144 mg. N/ml.

Mean: D_{25} sec.$^{-1}$ = 1·42, $D_{20} = 1{\cdot}24 \pm 0{\cdot}2 \times 10^{-6}$; mol.wt. = 11,000 ± 4000.

A certain amount of the high molecular weight precipitate had been removed from the digest used in the previous molecular-weight determination (Table 2). In view of this, it was desirable to determine the molecular weight in a filtrate from which as much as possible of the heat precipitate had been removed. This was done with filtrate 6.

Table 7. *Diffusion coefficient of filtrate*

Temp. (°)	Diffusion time (hr.)	Conc. in diffusate (20 ml.)	$D \times 10^6$
25	48	0·011	2·50
25	48	0·0108	2·55

Final conc. in cell, 0·273 mg. N/ml.

Mean: D_{25} sec.$^{-1}$ = 2·52, $D_{20} = 2{\cdot}18 \times 10^{-6}$; mol.wt. = 2000 ± 800.

It was heated at 80° for 30 min. before use and filtered, but very little additional precipitate formed. The filtrate was introduced into the diffusion cell, and the molecular weight was found to be somewhat lower than the previously determined value, but still clearly higher than the average peptide chain length in the filtrate.

Table 8. *Summary of Results*

	% of total N as Amino N	% of total N as Cystine N	Chain length	Mol. wt. (order of)
Precipitate (A)	8·2	12·7	10	11,000
(40%) (B)	8·7	13·2		(14,000)
Filtrate (60%)	16·2	7·9	5	2,000
				(1800)

DISCUSSION

The idea that a resynthesis of protein-like material takes place in the presence of pepsin under certain conditions was based on the observations that: (1) the proportion of nitrogen not precipitated by trichloroacetic acid (N.P.N.) is decreased, (2) the proportion of amino N in the precipitate is lower than that in the digest, and (3) that the phenomenon was not observed when boiled pepsin was added to the concentrated digests. In our experiments the formation of a precipitate from soluble pepsin digests occurred under circumstances in which the pepsin had been definitely inactivated. The pH and temperature are the main factors conditioning its formation. It is not produced at or about pH 2, but it is formed both in more acid and more alkaline solutions. The insoluble material appears to be identical with that precipitated by trichloroacetic acid. The fact that the proportion of amino N in the precipitate is less than in the digest as a whole cannot be cited as evidence of any resynthesis of peptide bonds, but merely means that the average chain length of the peptides in the precipitate is greater than that in the whole digest.

The general picture of the peptic digestion which we obtain is the breakage of about 11 peptide bonds in each submolecule of weight 12,000, giving a product which is initially soluble in the solvent in which it is formed and in the presence of trichloroacetic acid. Later, insoluble material is precipitated from this digest over a considerable range of pH and also in the presence of trichloroacetic acid. What is the nature of the reaction involved? The process is accelerated by heating, but occurs slowly even in the frozen state. It might be a hydrolytic reaction giving rise to an insoluble peptide, but there is no evidence that peptide bonds are split and moreover the effect of acidity does not support this explanation. We have also shown that there is probably no significant decrease of amino N during the formation of the precipitate, so that an appreciable resynthesis of peptide bonds is also ruled out.

The phenomenon seems to resemble that of protein denaturation. When insulin solutions are heated, insoluble denatured protein is formed by changes which do not involve the disruption of peptide bonds, but apparently merely a 'disordering' of the peptide chains which brings more hydrophobic material in contact with water and thus decreases solubility. It may be supposed that some of the larger protein fragments produced by pepsin may retain the structural configuration of the original insulin and the particular arrangement of the component groups, presumably with hydrophilic groups pointing outwards and hydrophobic groups inwards. Heating and addition of trichloroacetic acid might then produce a rearrangement or 'disordering' of these larger fragments, similar to that caused by heat on insulin itself, but occurring at a lower temperature. When the digest is exposed to pepsin for a longer time, the amount of insoluble substance which can be formed diminishes. This might be due to the original fragments being further split to smaller molecules which are no longer capable of this change. Table 8 summarizes the characteristics of the material in the precipitate and filtrate, so far as they have been determined. The molecular weight of the precipitated material is of the same order as that of the submolecule of insulin. It must therefore either be a more or less intact submolecule, or must be made up of portions of more than one submolecule. In the former case the percentage of amino N would be the same as insulin. The simplest interpretation of all the facts is that pepsin, like chymotrypsin, splits off terminal groups. The length of the peptide chains is not much greater than in the case of chymotrypsin, but they are aggregated in some way to give a diffusing molecule containing about three average peptide chains.

The residue of the insulin molecule is soluble when first formed, but undergoes changes on heating, etc., over a wide range of pH, except near pH 2. The residual molecule probably comes from more than one submolecule and may be derived from one half or more of the whole insulin molecule. The cystine is approximately equally divided between precipitated material and the soluble peptides. This possibly means that pepsin eats more deeply into the insulin 'core' than chymotrypsin in the initial stage of its action. Finally, we may observe that the plastein from egg albumin was shown by Folley (1932) to have a comparatively low molecular weight (<1000). It is likely that the mode of formation of this material, which requires more pepsin at pH 4, differs from that of the material from insulin, and the two phenomena are really dissimilar.

SUMMARY

1. The action of pepsin on insulin at pH 2 has been studied. Insulin is quickly and completely transformed into products which are not initially precipitated by trichloroacetic acid.

2. On standing at various pH values the digest undergoes changes whereby material is formed which is insoluble both in the presence of trichloroacetic acid and in the chosen buffer. This precipitate is similar to the so-called 'plastein', the formation of which has been observed in peptic and other digests.

3. The conditions of formation of the precipitate have been studied. No evidence has been obtained that it requires active pepsin for its formation, or that any appreciable resynthesis of peptide bonds occurs. It is suggested that the larger protein fragments originally formed retain much of the order of the protein, and may suffer changes similar to those which occur in protein denaturation.

4. The molecular weight of the precipitated material is of the order of 12,000 (chain length 10–12 residues), while that of the soluble split-products is c. 2000 (chain length about 5 residues). In both cases the diffusing molecule must be an aggregate containing several peptide chains.

One of us (J.A.V.B.) holds the Courtauld Research Fellowship in this Institute. Our thanks are also due to Messrs Courtauld Ltd. for a grant in aid of this investigation, and to the Imperial Chemical Industries for financial assistance. Sir J. Drummond, F.R.S., Research Director of Messrs Boots Pure Drug Co. Ltd., has very kindly supplied certain materials. We have also to thank Messrs Parke, Davis and Co. Ltd. for the supply of crystalline pepsin.

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The Biochemistry of the Gas Gangrene Toxins

3. DEVELOPMENT OF A MEDIUM SUITABLE FOR THE LARGE SCALE PRODUCTION OF THE TOXINS OF *CLOSTRIDIUM WELCHII* TYPE A

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(*Received* 23 *May* 1947)

In a previous paper (van Heyningen, 1941*b*) it was reported that, in order to obtain the toxins of *Clostridium welchii* type A, the organism was grown on a solution of commercial peptone supplemented with a protein-free extract of fresh muscle (Macfarlane & Knight, 1941). This medium has a low protein content which is desirable when the toxins are to be purified. Later it was necessary to produce the toxins on a scale of several thousand litres of culture filtrate. It was then found that the commercial peptone did not regularly produce good yields of toxin and accordingly another medium was developed. This medium consists essentially of papain digest of horse muscle pre-treated with alkali. In this work the main interest was in the production of the α-toxin (lecithinase).

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METHODS

(1) *Estimation of toxins*

α-Toxin was determined by measuring the combining power with specific antitoxin. 1 ml. quantities of a dilution of antitoxin containing 1 unit/ml. were pipetted into a number of tubes. Decreasing amounts of the toxin preparation, differing from each other by 10%, were added, and after standing for 30 min., 0·5 ml. of an egg-yolk solution (Macfarlane, Oakley & Anderson, 1941) was added. The tubes were placed in a water bath at 38°, and after 1 hr. readings were taken. The tube which contained the minimal amount of toxin free to react and cause turbidity contained one Le unit of lecithinase. The Le unit is that amount of toxin which in the presence of 1 unit of antitoxin contains a trace of free toxin; unless the toxin is considerably toxoided it is slightly larger than the Lf (flocculating) unit which is exactly neutralized by 1 unit of antitoxin. In fresh culture filtrates 1 Le is generally equivalent to about 20 egg units (E.U., van Heyningen, 1941*a*) or to about 60 average lethal doses (mouse, intravenous); these relationships are of course dependent upon the degree of toxoiding of the toxin. *θ*-Haemolysin was estimated in an analogous manner, with sheep red blood cells as substrate. The diluent was M/15-phosphate buffer, pH 7·4, 10^{-3} M with respect to thiolacetic acid, and containing enough α-antitoxin effectively to inhibit the α-toxin which is also haemolytic.

Collagenase. The *κ*-toxin (collagenase) antitoxin combining power of the filtrates was estimated by the method of Oakley, Warrack & van Heyningen (1946).

(2) *Other estimations*

Total N was determined by micro-Kjeldahl, amino N by the copper method of Pope & Stevens (1939). Iron was determined colorimetrically with αα′-dipyridyl. pH was determined electrometrically with a dipping glass electrode.

EXPERIMENTAL

A number of enzyme digests of fresh and old (1 month in a refrigerator) horse muscle and of casein were prepared, with hog stomach and commercial pepsin, pancreatic extract and commercial trypsin, and commercial papain as the sources of proteolytic enzymes. Variations were made in the time and temperature of digestion and in the ratios of non-dialyzable and amino N to total N. Supplements such as protein-free muscle extract and yeast extract were added to these digests, but in no case was a satisfactory yield of toxin obtained from cultures grown on media made from the digests, although the organism always grew profusely. Occasionally a non-reproducible yield of about 1 Le/ml. was obtained. Then it was discovered that if the meat was treated with alkali before digestion, and if the digestion was carried out with the meat in high concentration, a medium could be made upon which satisfactory yields of toxin (3–5 Le/ml.) could be obtained.

Effect of alkali on pH *of meat.* 2 kg. quantities of finely minced and defatted meat were mixed with 1 l. quantities of distilled water to which had been added various volumes of 2 N-NaOH ranging from 60 to 140 ml. The mixing was done in a Hobart Dough Mixer at high speed. It was essential to use this type of mixer which has a paddle rotating with a planetary motion, otherwise it was not possible to get a uniform pH throughout the comparatively stiff meat-water paste. After 15 min. mixing the pH of the meat was determined and the meat then stored at 2–4°. During this time the pH slowly fell, as is shown in Fig. 1. Curve *A* shows the pH of the mixtures immediately after the 15 min. mixing, curve *B* after 2 hr. and curve *C* after 14 hr. in the cold room. After 14 hr. there was very little further change in the pH. The values shown in curve *A* were not closely reproducible even if meat of the same initial pH was used, but it was found that the amount of toxin subsequently produced from digests of the meat was dependent not so much upon the pH to which the meat was adjusted as upon the amount of alkali added to the meat.

After the various samples of meat had stood for 48 hr. at 2–4° 1 g. of commercial papain (Thomas Morson) was mixed with each and the temperature was raised to 60° and kept there with occasional stirring for 3·5 hr. The meat rapidly liquefied. After the digestion the pH was lowered to 5·5 and the digests were boiled for 10 min. and filtered. These filtrates (*c.* 1250 ml./2 kg. meat) generally contained about 15 g. total N/l., 3 g. amino N/l., 2 g. non-dialyzable total N/l., and 10–13 mg. available Fe/l. The available iron was decreased to 200–300 μg./l. by adjusting the pH to 7·8, adding 3 mmol. $CaCl_2$/l., boiling and filtering. The filtrates were then diluted to contain 6 g. total N/l., 1% $FeSO_4 . 7H_2O$ was added to bring the available iron concentration up to 1200 μg./l. and the following reagents added/l.: 7·5 g. sodium glycerophosphate; 6 g. glucose; 0·1 ml. thiolacetic acid. The pH was adjusted to 7·2 and the medium sterilized by filtration. Each batch of medium was inoculated with a fresh culture of *Cl. welchii*, type A, strain S 107, and incubated at 37° for 5 hr. The cultures were then filtered and the α-toxin content of the filtrates determined. The results are plotted in Fig. 2. The data in this curve are reproducible in that optimum toxin production is always obtained when 80 ml. N-NaOH are used for every 2 kg. meat, but the value 4·72 Le/ml. is higher than average; generally 3–3·5 Le/ml. are obtained. If the pH of the meat rather than the amount of alkali added were plotted on the abscissa the curve would not be reproducible. An attempt was made to prevent the pH of the meat from changing while standing at 2–4°, by mixing in $CaCO_3$, but no improvement in toxin production was observed. It was also found that there was no change in toxin production if the alkali-treated meat was allowed to stand for as long as 6 days. Toxin production was always slightly lower if the meat stood for only 1 day.

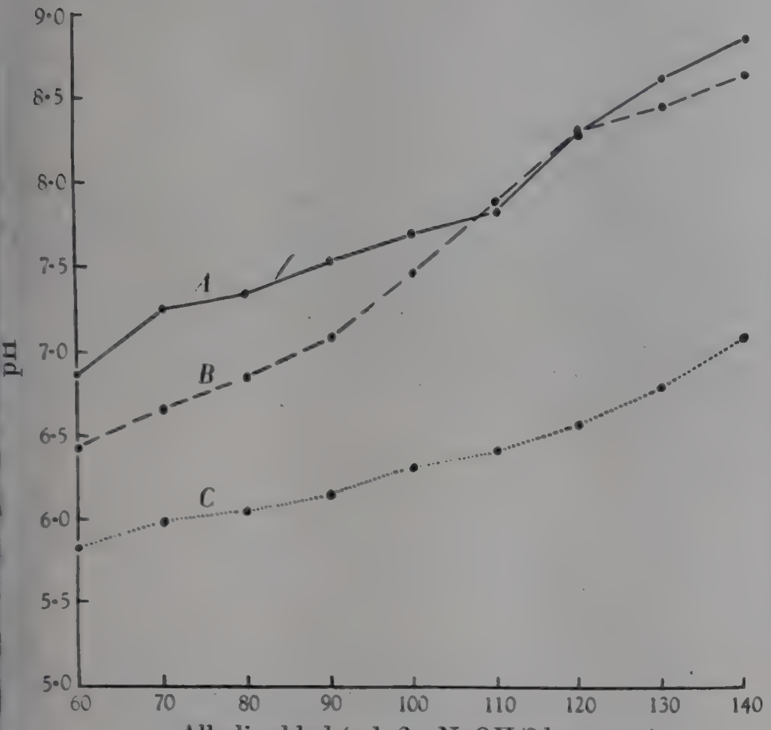

Fig. 1.

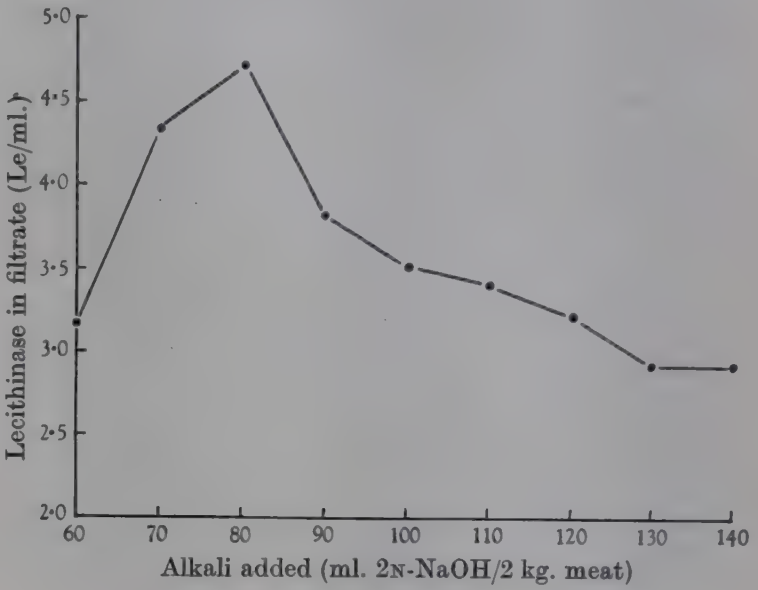

Fig. 2.

Fig. 1. Effect of alkali on the pH of meat. Time at 2–4° after addition of alkali: *A*, 15 min.; *B*, 2 hr.; *C*, 14 hr.

Fig. 2. Effect of alkali during pre-treatment of meat, on yield of lecithinase.

It is important that the digestion of the meat should be carried out with the concentration of meat as high as 2 kg./l. of added water. When larger quantities of water were used the toxin production was invariably lower; with a ratio of meat to water of 1:1 the toxin production was less than half.

It is an advantage to add the papain to the meat while the alkali is being mixed in; this does not affect toxin production, but it simplifies the digestion procedure. If papain is initially present the meat starts liquefying while the temperature is being raised to 60° for the digestion, and is consequently more easily manipulated and stirred than it would be without papain. Without the papain there is a tendency for the meat to overheat. When crude pepsin and pancreatic extract were used instead of papain a medium was obtained upon which about half as much toxin could be obtained as on a papain digest.

The addition of protein-free horse-muscle extract (Macfarlane & Knight, 1941) to media prepared in this way did not increase toxin production, in fact it always had a slight inhibiting effect.

Pappenheimer (private communication; cf. Pappenheimer & Shaskan, 1944) had found that with a medium he was using the concentration of available iron had a marked effect on α-toxin production. The concentration of available iron was also found to influence the α-toxin production in the deferrated medium described above, but not to a great degree, as will be seen in Fig. 3. There appears to be an optimal iron concentration at 1000–1200 μg./l. Later it was found that if the medium was not deferrated it was not affected by the concentration of available iron, and as high concentrations of toxins could be produced on such media, containing about 4000 μg. available Fe/l., as could be obtained on deferrated

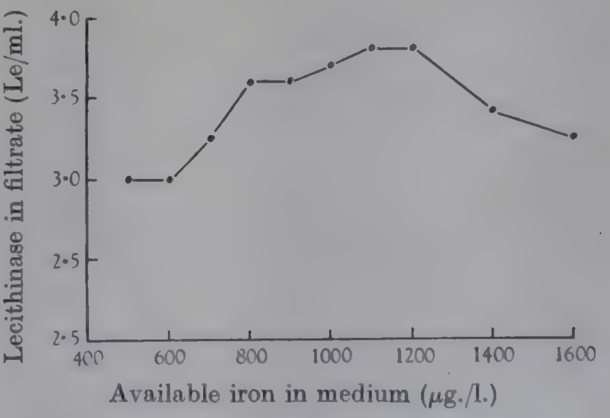

Fig. 3. Effect of iron, added to deferrated media, on yield of lecithinase.

media to which iron was added to the optimal concentration of 1200 μg./l. It is possible that during deferration some substance or substances which renders the organism less susceptible to the available iron is removed in addition to the iron.

DISCUSSION

On a medium produced in the laboratory by the methods described above culture filtrates containing 3·5–5 Le units of lecithinase/ml. can be obtained with satisfactory regularity. The yield of θ-haemolysin varies from 2 to 15 units/ml., and that of collagenase between 4 and 6 κ units/ml. The medium has successfully been produced on the large scale by Mr J. G. C. Campbell of these laboratories with such modifications as production procedures entail. During 12 months more than 6000 l. of this medium were produced, generally in batches of 200–300 l., for the production of lecithinase. The yield of lecithinase was generally between 2·5 and 4 Le/ml.

It is not yet known why the meat should have to receive the alkali treatment before digestion, or why the digestion should have to be carried out with a high concentration of meat. It is possible that during the period of standing, during which the pH passes through neutrality, autolysis takes place under the conditions observed and toxigenic substances are set free. Rogers & Knight (1946) have shown that autolysis of meat favours the production of substances which stimulate lecithinase production by *Cl. welchii*. The high concentration of meat during digestion might be necessary in order that an adequate concentration of toxigenic substances may be extracted from the meat in relation to the concentration of other nutrient and toxigenic substances liberated by the digestion.

An empirical medium such as the one described in this paper is obviously unsatisfactory in the long run. It savours more of the kitchen than of the laboratory, since there are a number of uncontrollable and unknown factors in its production and in its constitution. The development of a medium of which the constituents are known is obviously desirable.

SUMMARY

The development of an empirical medium suitable for the large-scale production of the α-toxin (lecithinase) of *Clostridium welchii*, type A, is described.

I am grateful to Mr R. N. Beale and to Miss E. Bidwell for much assistance and to Dr B. C. J. G. Knight and Mr J. G. C. Campbell for helpful discussion and advice.

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The Biochemistry of the Gas Gangrene Toxins

4. THE REACTION BETWEEN THE α-TOXIN (LECITHINASE) OF *CLOSTRIDIUM WELCHII* AND ITS ANTITOXIN

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(*Received* 23 *May*, 1947)

If constant amounts (300 units) of a sufficiently avid diphtheria antitoxin (horse serum) are added to a series of tubes containing increasing amounts of diphtheria toxin, ranging from 150 to 450 Lf units, flocculation will take place in each tube and the supernatants will be very nearly immunologically neutral. The ratio of toxin to antitoxin in the floccules will vary from TA_2 to T_3A_2, where T is an Lf unit of toxin and A is a unit of antitoxin. If. (*a*) no other antigen-antibody systems are involved in the flocculation, (*b*) no impurities associated with the toxin or antitoxin preparations are adsorbed on the floccules, and (*c*) the toxin-antitoxin floccules are quantitatively precipitated, then the floccules should contain all the toxin and all the antitoxin added to each tube, and nothing else. The difference between the nitrogen contents of the floccules from the mixtures containing 150 and 450 toxin units will represent the N content of 300 toxin units. In other words, if the N content of the floccules is plotted on the ordinate against the units of toxin employed on the abscissa, a straight line will be obtained whose slope will represent the N content of one unit of toxin and whose intercept with the ordinate will represent the N content of 300 units of antitoxin.

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By employing a technique involving the principles outlined above Pappenheimer & Robinson (1937) concluded that the N content of 1 Lf unit of diphtheria toxin is 0·00046 mg., and of 1 unit of antitoxin is 0·0016 mg. This figure of 0·00046 mg. N/Lf agrees very well with the figure of 0·00045 mg. N/Lf which Pappenheimer (1937) later found by direct estimation for pure diphtheria toxin. Since the figure given by Pappenheimer & Robinson was obtained irrespective of the purity of the toxin and antitoxin preparations* they used, we had hoped, in the course of our work on the purification of the α-toxin of *Clostridium welchii*, to employ a similar technique for determining the percentage specifically precipitable N, i.e. the percentage purity, of our toxin preparations. Our experience shows, however, in the case of *Cl. welchii* α-toxin at any rate, that nonspecific impurities are adsorbed on the toxin-antitoxin floccules. We have also observed that different batches of toxin behave differently in the toxin-antitoxin reaction.

METHODS

Estimation of toxin and antitoxin. Toxins and antitoxins were estimated either by measuring their antitoxin and toxin combining power (Le/ml. for toxins) with crude lecithin as indicator for slight toxin excess (see van Heyningen, 1948), or by measuring their flocculating power (Lf/ml. for toxins). For most of the work the latter method was used. The primary standard was the British Standard Perfringens Antiserum (National Institute for Medical Research). The Lf units/ml. of a toxin preparation, Pf 13D, were determined against this standard, and a substandard antitoxin preparation, RX 5167 (which was pepsin-refined and which was also the source of antitoxin used in all the experiments described in this paper) was assayed against it. All other toxin preparations were assayed against the substandard antitoxin preparation. In all assays increasing amounts of antitoxin were mixed with constant amounts of toxin ('β-procedure') and the order of flocculation observed. If the flocculations are carried out at 40° rather than 50° a better accuracy is obtained, since the time intervals at which the different mixtures flocculate are longer. Conditions were so arranged that the indicating tube contained about 40 units each of the reactants in a volume of about 2·5 ml., and that the mixture flocculated in about 7 min.

For each assay at least 25 tubes in duplicate were used in which the amounts of antitoxin differed by 2 % increments. The tubes were compared five (sometimes seven) at a time according to the scheme below:

A	B	C	D	E
1	6	11	16	21
2	7	12	17	22
3	8	13	18	23
4	9	14	19	24
5	10	15	20	25

The serial numbers represent tubes in which the amounts of antitoxin increase by 2 %; the horizontal rows of numbers represent the sets of tubes observed at one time.

Each set of five tubes was set up in duplicate. Thus five duplicate observations were made in which the spacing between the successive amounts of antitoxin was 10 %, the final effect being that the relative order of flocculation of mixtures containing antitoxin with 2 % spacing was obtained. According to the order of flocculation of three adjacent mixtures, B, C and D, the amount of antitoxin which was considered equivalent to the constant amount of toxin used was judged as follows:

Order of flocculation	Equivalent amount of antitoxin
C, then B and D simultaneously	C
B, C and D simultaneously	C
C and D simultaneously	Mean of C and D
B and C simultaneously	Mean of B and C
C, then D	C + 25 % of difference between C and D
C, then B	C − 25 % of difference between C and B

By this means values with an error of ± 2 % or less could be obtained 19 times out of 20. Thus the value of the toxin preparation Pf 13D was found to be 1234 ± 11 Lf/ml., and that of the antitoxin preparation RX 5167 was 4462 ± 73 units/ml.

Diffusion coefficients. The diffusion coefficient of α-toxin and of the pigment associated with it in some preparations was measured by the method of Northrop & Anson (1929). The enzyme and the pigment were allowed to diffuse at 3·5° into borate buffer pH 6·7, and the Le or pigment content was measured at daily intervals. Two diffusion cells were used, one of which was kindly lent to us by Dr Roy Markham. The constants for the cells were determined with carbon monoxide haemoglobin, and were 0·0641 and 0·378. On the advice of Dr Markham the diffusion coefficient, D_{20}, of CO haemoglobin was taken as 0·0553 cm.^{2}/day.

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* Pappenheimer & Robinson (1937) used both crude and pepsin-refined antitoxic globulin; the N content/unit of the latter was lower than the former. See also Pope & Healey (1939).

Other estimations. Nitrogen was determined by micro-Kjeldahl procedure and pH was determined electrometrically with a glass electrode.

EXPERIMENTAL

Partial purification of α-toxin

The toxin preparations used were derived from filtrates of cultures of *Cl. welchii* type A, strain S 107, grown on the medium described by van Heyningen (1948).

Purification by ammonium sulphate fractionation. Concentrated and partially purified (at most 10% pure) preparations were routinely obtained from the crude filtrates according to the steps outlined in Table 1. The figures in Table 1 are the means from 22 experiments, and in each case they have been brought to the nearest round number.

Table 1. *Partial purification of α-toxin by ammonium sulphate fractionation*

Preparation	Volume (l.)	Treatment	Le*/ml.	Overall yield (%)	Le*/mg. non-dialysable N	Overall purification
1 (crude filtrate)	200	Stir in 120 kg. $(NH_4)_2SO_4$. After standing overnight pick off the firm scum from the surface, dissolve in water and filter. pH 6·5	3	—	5	—
2	2	Dialyze 3 days against running tap water	240	80	15	3-fold
3	4	Stir in 1·68 kg. $(NH_4)_2SO_4$. After 1 hr. collect precipitate and dissolve in water. pH 6·5	114	76	60	12-fold
4	0·50	Dialyze overnight against running tap water	820	69	120	24-fold
5	0·75	Add 0·8 vol. saturated $(NH_4)_2SO_4$ solution. After 1 hr. collect precipitate and dissolve in water. pH 6·5	520	65	120	24-fold
6	0·25	Store or use	1150	49	250	50-fold

* Le = lecithinase antigen units.

The final product, of which preparation Pf 13 D is an example, was highly pigmented—so much so that it was impossible to examine it in the electrophoresis apparatus. The yield and degree of purification obtained by this procedure were much the same as those obtained by an earlier procedure which involved adsorbing the toxin on calcium phosphate in the presence of acetone (van Heyningen, 1941). The present procedure can more conveniently be applied to large volumes of culture filtrate. Further treatment of the final product with $(NH_4)_2SO_4$, organic solvents, or adsorbents failed to yield a purer preparation. Preparations such as these were used for the production of concentrated toxoids in an investigation of the possibilities of active immunization against gas gangrene. For convenience these preparations will be called AS preparations.

Purification by nucleic acid precipitation. The Warburg school have recently used nucleic acid with great success in the isolation of enzymes from yeast (Warburg & Christian, 1939) and from tumour tissue (Kubowitz & Ott, 1943). We therefore applied this reagent to the purification of α-toxin. The starting material was preparation 3 of Table 1, i.e. the dialyzed solution of the $(NH_4)_2SO_4$ precipitate of the toxin from the crude filtrate. It was found to be essential that the preparation should be dialyzed. It was then treated in the following way:

(1) Crude toxin solution (4 l.) was cooled to 2–3° and held there. A neutral solution containing 4 g. yeast nucleic acid was added, and the solution carefully adjusted to pH 4·5 by adding about 320 ml. N-acetic acid over a period of 1 hr. with stirring. The acidified solution was left to stand at 2–3° for 2 hr. during which time a small dark brown precipitate settled out and was collected. The supernatant solution contained most of the κ-toxin (collagenase, see Bidwell & van Heyningen, 1948) and was therefore readjusted to pH 7 and stored. The precipitate was suspended in 40 ml. water and N-NaOH carefully added with stirring until the pH was 6·0; the precipitate then dissolved completely to give a very dark brown solution containing the α-toxin in 50% stage yield. The solution contained nucleic acid, some of which presumably was bound to the toxin.

(2) Nucleic acid, and probably other impurities, can be removed by the addition of protamine until no further precipitate is obtained. It is not possible to calculate how much protamine to use, and therefore it was necessary to carry out a preliminary titration on a sample of the toxin-nucleic acid solution. The amount of protamine sulphate required to work up 200 l. of crude filtrate varied from 1·5 to 4 g. It was added in the form of a 2% solution of salmine sulphate (Wellcome Chemical Works) at pH 6. At this pH the solution was very opalescent and probably some of the protamine was carried in suspension. A considerable amount of pigment was removed by the protamine pre-

cipitation, leaving a light yellow supernatant solution containing the α-toxin in 60–80% stage yield.

(3) After the protamine nucleate precipitate had been removed by centrifugation excess protamine and some other impurities, including pigment, were removed by treating the solution with 1/8th volume of 30% alumina cream suspension (i.e. 30% solid by volume). The stage yield of α-toxin was 75%, and the overall yield from crude filtrate to this stage about 20%. The final product was nearly white when dry and light yellow in 2% solution. Upon electrophoresis (kindly done for us by Mr P. A. Charlwood) the preparation appeared to contain four components, the most abundant of which amounted to about 50% of the total solids. These preparations contained 1200–1500 Lf/mg. N, and about 10,000 average lethal doses (mouse, intravenous)/mg. As will be seen later, these preparations cannot be more than 50% pure; the overall purification with respect to crude filtrate was 250-fold, or 5-fold with respect to the AS preparations previously mentioned. Compared with crude filtrate the ratio of κ-toxin to α-toxin in the nucleic acid purified preparations was decreased about 30-fold; the hyaluronidase/α-toxin ratio was decreased about 2000-fold; the θ-toxin/α-toxin ratio remained almost unchanged. For convenience these preparations will be referred to as NA preparations. The diffusion coefficient of the α-toxin, D_{20}, was found to be 0·064 cm.²/day. We were not tempted to calculate the molecular weight of the toxin from its diffusion coefficient.

THE TOXIN-ANTITOXIN REACTION

The diphtheria toxin-antitoxin reaction has been studied by three techniques:

(i) Pappenheimer, Lundgren & Williams (1940) made an ultracentrifugal study of the toxin-antitoxin complexes in solution in excess toxin and antitoxin. From the sedimentation characteristics of the components formed in these regions they deduced the existence of complexes ranging from TA_4 to T_4A. (T and A represent units of toxin and antitoxin respectively. The expression TA_4, to take an example, does not necessarily represent a single complex, but the unit ratio of toxin to antitoxin in a mixture of, say, two parts of TA_5 and one part of TA_2. It is in this sense that the expressions T_xA_y are used throughout this paper.)

(ii) From determinations of the residual toxin or antitoxin contents of mixtures of toxin and antitoxin, in which precipitation may or may not have occurred, Healey & Pinfield (1935) concluded that complexes ranging from TA_2 to T_3A_2 were formed. They were not able to detect complexes containing more antitoxin than in TA_2, or more toxin than in T_3A_2. Evidently antitoxin in excess of the ratio TA_2 reacts rapidly with added toxin to form TA_2, whereas TA_2 reacts slowly with T to form TA.

Thus
$$TA_4+T \xrightarrow{\text{fast}} 2TA_2,$$
$$TA_2+T \xrightarrow{\text{slow}} 2TA$$
and similarly
$$3T_4A+5A \xrightarrow{\text{fast}} 4T_3A_2,$$
$$T_3A_2+A \xrightarrow{\text{slow}} 3TA.$$

(iii) By determining the nitrogen in the floccules precipitated from toxin-antitoxin mixtures Pappenheimer & Robinson (1937) made a quantitative study of the formation of insoluble toxin-antitoxin complexes.

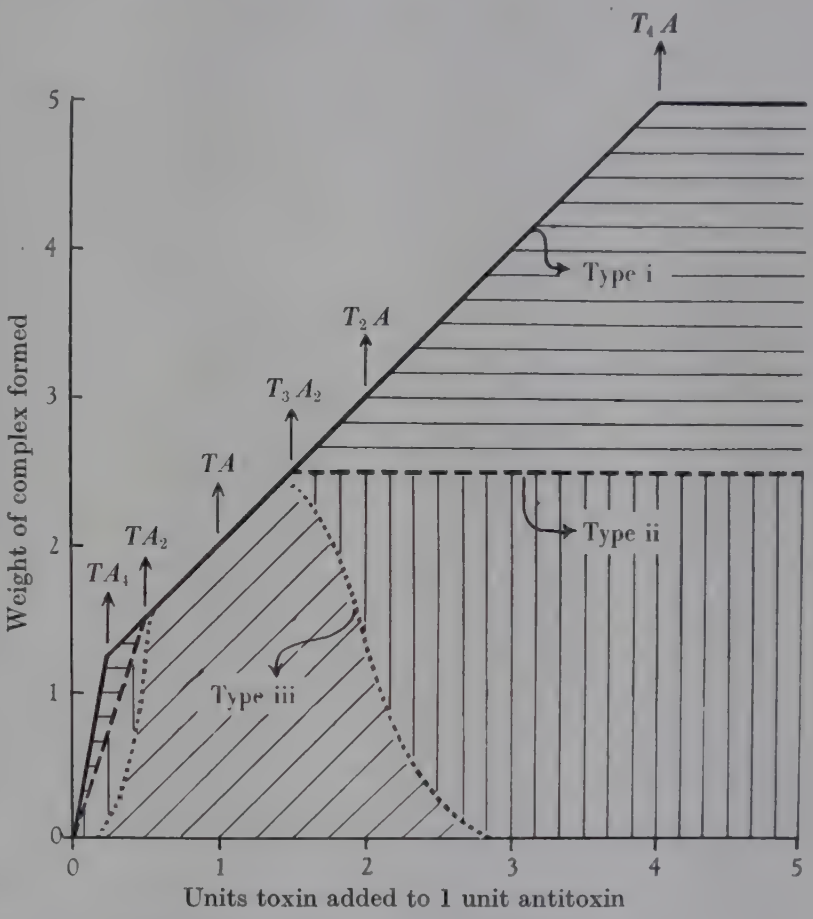

Fig. 1. Weights of complexes formed by interaction of toxin and antitoxin of arbitrary unitage and weight. Type i, total complexes; type ii, soluble and insoluble slow-reacting complexes; type iii, insoluble complexes. (See text for explanation.)

The information gained from the application of these three techniques is illustrated in Fig. 1, in which are plotted the weights of toxin-antitoxin complexes formed, using arbitrary units and taking a weight of 1 for each unit. The type of graph obtained by each technique is correspondingly marked; the horizontally hatched areas show the soluble toxin-antitoxin complexes which react rapidly with toxin or antitoxin to form the complexes shown in the vertically hatched areas; the vertically hatched areas show the soluble complexes which react slowly; the diagonally hatched area shows the insoluble complexes, which also react slowly.

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We have made a similar study of the *Cl. welchii* α-toxin-antitoxin reaction with the latter two techniques ((ii) and (iii) above).

Titration of supernatants

Increasing amounts of the toxin preparation Pf 13D were mixed with constant amounts (242 units) of antitoxin, in a constant volume of 6 ml. After 3 days standing at 4° the floccules which precipitated in all the mixtures were spun down and the supernatants titrated for toxin and antitoxin with egg yolk as indicator. The results obtained are shown in Table 2. From these figures it appears that with the preparation Pf 13D complexes ranging from TA_2 to T_2A are detectable. The preparation Pf 13D is exceptional, however; with most batches of toxin the range is approximately T_2A_3 to T_6A_5, i.e. the ratio of toxin to antitoxin in the complexes covers about a twofold range. Mixtures of toxin and antitoxin were made containing either 12,000 units of antitoxin and 3000 units of toxin, or 12,000 units of toxin and 4000 units of antitoxin, in 180 ml. volumes, involving altogether 10 different batches of toxin and one batch of antitoxin. In none of these mixtures was there any precipitate formed. After standing overnight at 4° the free antitoxin or toxin was determined. The titrations showed that on the antitoxic side the complexes detected varied from $TA_{1\cdot45}$ to $TA_{1\cdot61}$, and on the toxic side from TA to T_3A_2, most of the toxic complexes having the composition T_6A_5.

Table 2. *Titrations of free antitoxin or antitoxin in mixtures containing increasing amounts of toxin and 242 units of antitoxin*

I Toxin added (units)	II Free antitoxin (A) or toxin (T) in supernatants (units)	III Units antitoxin (A) combined with units toxin in col. I or units toxin (T) combined with 242 units antitoxin	IV Ratio T/A in complex	
95	72 (A)	170 (A)	0·55	
119	6·5 (A)	236 (A)	0·50	
166	1·2 (A)	241 (A)	0·69	
214	0·3 (A)	242 (A)	0·88	
242	<0·01 (T)	242 (T)	1·00	
363	<0·01 (T)	363 (T)	1·50	
484	<0·01 (T)	484 (T)	2·00	Mean 1·98
605	144 (T)	461 (T)	1·90	
726	264 (T)	462 (T)	1·91	
847	396 (T)	451 (T)	1·86	
965	432 (T)	533 (T)	2·20	

From the work of Healey & Pinfield (1935) it is evident that complexes of the type TA_2 react slowly with toxin to form TA. We have found that α-toxin-antitoxin complexes will react in a similar manner, but that again there is variation with different batches of toxin. Mixtures were made containing 12,000 antitoxin units and 3000 toxin units in a volume of 180 ml. After standing overnight at 4° the free antitoxin was determined as follows: increasing amounts (spaced 10%) of the antitoxic mixtures were added to constant amounts (1 Le unit) of test toxin. Several similar series of such mixtures were made. Pairs of these series were left to stand for 30 min. (as in the usual assay), 1 day and 2 days before the egg-yolk solution was added and the mixtures incubated at 37° for 1 hr. The longer the antitoxic mixtures were allowed to stand with the test toxin the more toxin they combined with, i.e. the greater was their apparent antitoxin content. The results obtained with three different batches of toxin are shown in Table 3. Batches B 195G and B 197G behaved much as the samples of diphtheria toxin studied by Healey & Pinfield (1935). The interesting behaviour of batch B 202J

Table 3. *Slow reaction between T and complexes containing more A than in ratio $T/A = 1$*

Toxin	Units toxin added to 12,000 units antitoxin	Apparent free antitoxin units on 30 min. test	Apparent units antitoxin combined	Ratio T/A in complex detected on rapid T-A reaction	Apparent free antitoxin units on 2-day test	Apparent units antitoxin combined	Ratio T/A in complex detected on slow TA_x-T reaction
B 195G	3,000	7,650	4,350	0·69	9,370	3,630	0·83
B 197G	3,170	6,800	5,200	0·61	8,500	3,500	0·91
B 202J	3,000	7,650	4,350	0·69	10,500*	1,500	2·00

* 1-day test.

will be referred to again in the discussion. It would have been interesting to study the reaction of complexes containing higher ratios of toxin than TA, and batch Pf 13D with which a ratio of T_2A was detected would have been very suitable; unfortunately, it had all been used by the time we realized that these experiments should be done. With the other batches of toxin complexes of average composition T_6A_5 were detected and the difference between T_6A_5 and TA is too small to allow significant changes to be observed.

Quantitative flocculation

The optimum conditions for quantitative flocculation were determined.

Effect of temperature. Mixtures containing equivalent amounts of toxin and antitoxin made up to 6 ml. with 1 % NaCl were (*a*) left at 4° for 4 days, (*b*) left at 4° for 4 days, the floccules then resuspended, kept at 50° for 3 hr., and then left at 4° for another day, (*c*) kept at 50° for 3 hr., then left at 4° for 4 days. The floccules were then spun down in a refrigerated centrifuge, washed twice with 6 ml. chilled saline and their N content measured. The results of duplicate determinations are shown in Table 4. Since no significant differences resulted from the different temperatures at which the mixtures were kept, mixtures hereafter were always left in the cold room, without treatment at 50°.

Table 4. *Effect of temperature on flocculation*

Units toxin and antitoxin	Nitrogen in floccules (mg.) 4 days at 4°	 4 days at 4°, 3 hr. at 50°, 1 day at 4°	 3 hr. at 50°, 4 days at 4°
100	0·296	—	0·281
	0·278	0·307	0·261
200	0·616	0·612	0·597
	0·625	0·597	0·598
300	—	—	0·932
	0·934	0·907	0·945
400	1·236	1·232	1·236
	1·281	—	1·227
500	1·600	1·549	1·586
	1·591	—	1·528

Effect of concentration of reactants on flocculation. Mixtures containing 190 Lf units of toxin and 242 units of antitoxin were made up to different volumes and left at 4° for 5 days. The floccules were then washed twice with 6 ml. chilled saline and their N content determined. The results are shown in Table 5. It will be seen that within a range of nearly threefold the concentration of toxin-antitoxin does not significantly affect the amount of precipitate formed.

Table 5. *Effect of concentration on flocculation*

Volume (ml.)	3	4	5	6	7	8
Nitrogen in floccules (mg.)	0·783	0·757	0·751	0·756	0·825	0·744
	—	0·761	0·754	0·767	—	0·748

Effect of time of standing and of washing on floccules. Identical mixtures containing 190 Lf units of toxin and 180 units of antitoxin made up to 6 ml. in saline were left at 4° for different times, and then the floccules were spun down and washed 2 or 3 times with 6 ml. volumes of chilled saline. The results are shown in Table 6. They suggest that possibly there was a very slight increase in the amount of N precipitated in 3 days as compared with 1 day. They do not show any significant difference between two and three washings (see also Table 9).

The quantitative study of the flocculation reaction was carried out as follows: Appropriate amounts of toxin and antitoxin were carefully pipetted into 15 ml. centrifuge tubes, thoroughly mixed, and the volumes made up to 6 ml. with 1 % NaCl solution. The tubes were then fitted with rubber caps and left at 4° for 4 days. Then the floccules were spun down in a refrigerated centrifuge, resuspended in 6 ml. chilled saline with the help of a small high-speed stirrer, spun down and washed a second time. After being spun down for the last time the floccules were Kjeldahl-digested in their centrifuge tubes.

Table 6. *Effect of time of standing, and of washing on amount of floccules obtained*

Time (days)	...	1	2	3	5	6
Nitrogen in floccules (mg.)	2 washings	0·596	—	0·615	0·626	0·560
		0·601	—	0·612	0·637	0·633
	3 washings	0·595	0·596	0·600	0·627	0·602
		—	0·593	0·589	0·627	0·613

The experiments done with toxin preparation Pf 13D are typical. Increasing amounts of toxin, varying from 95 to 965 Lf units, were mixed with constant amounts (242 units) of antitoxin and the N content of the floccules determined. The results are plotted in Fig. 2. (The point corresponding to

95 toxin units is the mean of four determinations, the points in the equivalence zone from 118·5 to 237 toxin units are each the mean of ten determinations; the points in the range 363–965 toxin units are each the mean of four determinations.) The mean values for the equivalence zone are given in Table 7. In the equivalence zone the supernatants contain neither free toxin nor free antitoxin. The points in the equivalence zone lie on a straight line, the slope of which is the amount of nitrogen associated with one Lf unit of toxin, and the intercept of which with the ordinate is the amount of nitrogen associated with the total number (242) of antitoxin units used. These relationships are given by the expression $y=a+bx$, where $y=\text{N}$ in floccules, $a=\text{N}$ associated with antitoxin in floccules, $b=\text{N}$ associated with 1 toxin unit in the floccules, and $x=$ number of toxin units.

In all the experiments of this type we have calculated the slope (b) of the best straight line passing through the points in the equivalence zone, and from that its intercept (a) with the ordinate, by the usual statistical methods. For convenience the figure for the amount of N associated with one Lf unit of toxin in the floccules will be called the 'slope' of a given toxin preparation, and the corresponding figure for the amount of N associated with one unit of antitoxin will be called the 'intercept'. Thus for the toxin preparation Pf 13D the 'slope' was found to be 0·00201 mg. N/Lf toxin, and the 'intercept' was found to be 0·00146 mg. N/unit antitoxin.

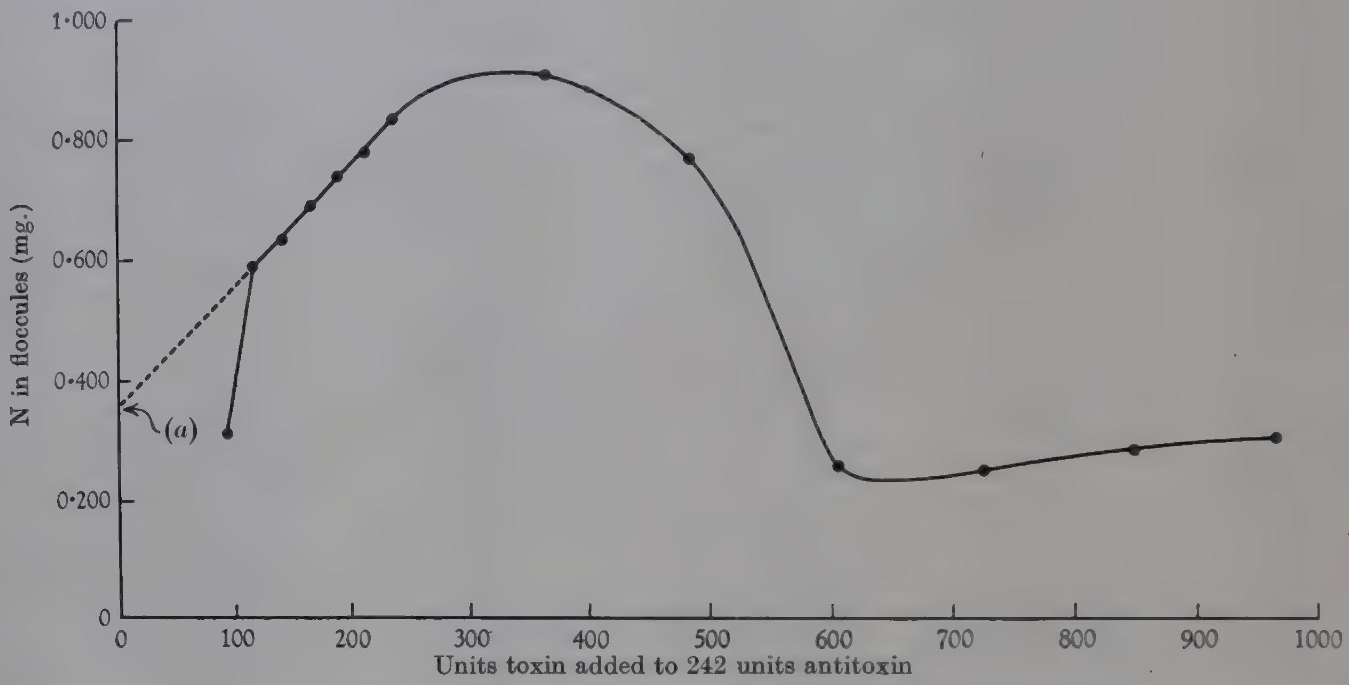

Fig. 2. Nitrogen in floccules precipitating from toxin-antitoxin mixtures.

Fig. 3 shows that essentially the same curves were obtained when the nitrogen in the floccules was either determined directly, or calculated from the difference in the N content of the toxin-antitoxin mixtures before and after flocculation. (Each point is the mean of three or four determinations.) The values found by difference were consistently slightly higher than those found directly.

The curves in Figs. 2 and 3 are much the same as that found for diphtheria toxin by Pappenheimer & Robinson (1937). The main difference between our curves and theirs is that we still found appreciable

Table 7. *Mean values for equivalence zone in Fig. 2*

I x Units toxin	II Ratio A units/T units	III bx Toxin N (mg.)	IV y Ppt. N (mg.)	V a Antitoxin N in ppt. (mg.) (IV – III)
118·5	2·04	0·239	0·593	0·354
142·5	1·70	0·286	0·638	0·352
166	1·46	0·334	0·689	0·355
190	1·27	0·382	0·737	0·355
213·5	1·14	0·430	0·784	0·354
237	1·02	0·478	0·830	0·352

precipitates when the ratio of toxin to antitoxin units was as high as 4 : 1, whereas in the diphtheria system there was no precipitate beyond the ratio 2 : 1. Pope & Healey (1938) point out that diphtheria antitoxin modified by treatment with pepsin (Pope, 1938) flocculates over a wider range than does untreated antitoxin.

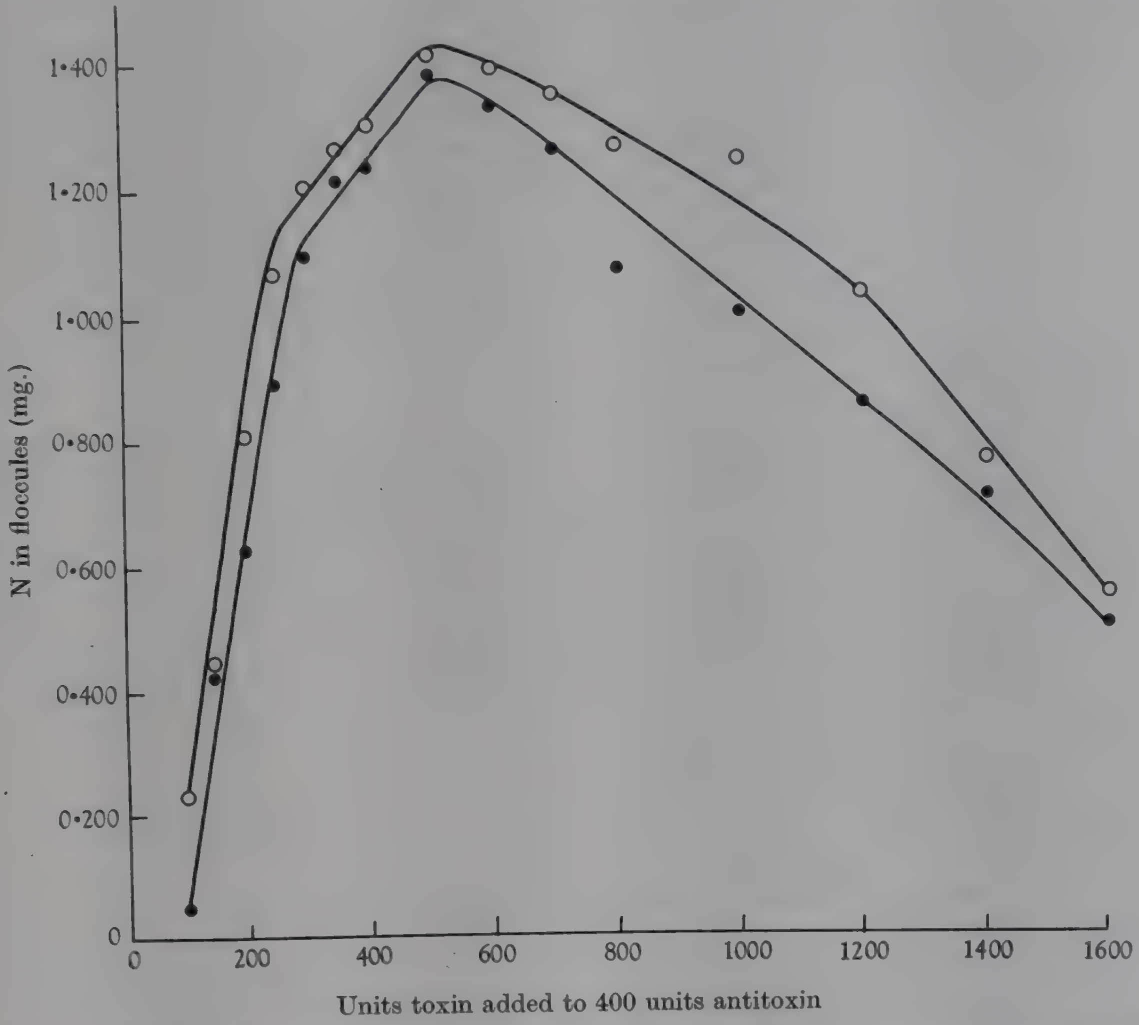

Fig. 3. Nitrogen in floccules precipitating from toxin-antitoxin mixtures. Upper curve, as deduced from difference in N contents of mixtures before and after flocculation; lower curve, as determined directly on the floccules.

Table 8. *Variations in 'intercepts' with different batches of AS toxin preparations*

Toxin preparation	'Slope' N associated with 1 Lf unit toxin (mg.)	'Intercept' N associated with 1 unit antitoxin (mg.)
B 195G	0·00185	0·00194
B 165G	0·00184	0·00184
Pf 13D	0·00201	0·00146
B 170G	0·00180	0·00080
B 197G	0·00079	0·00119

The 'slope' and 'intercept' for Pf 13D were determined at least five times, and the values obtained were never significantly different from 0·0020 and 0·00146 mg. N respectively; but when similar determinations were made on a number of different batches of AS toxin preparations we found that, although most of them gave approximately the same 'slopes', the 'intercepts' varied from batch to batch. Table 8 shows the values obtained for five batches of AS toxin preparations. Batch B 197G is exceptional. That these differences in 'intercepts' are not due to batch to batch variations in the solubility of the floccules in the saline wash liquors is evident from the figures in Table 9. For each of ten different batches of AS toxin preparations eight

Table 9. *Showing insolubility of floccules in saline*

Toxin preparation	N in floccules (mg.)	
	Washed with 12 ml. saline	Washed with 40 ml. saline
B 158G	1·451	1·299
B 165G	1·328	0·966
B 166G	1·323	1·286
B 170G	1·315	1·007
B 174F	1·299	1·052
B 146A	1·233	1·309
Pf 199	1·126	1·299
Pf 219	1·025	1·186
B 195G	0·991	1·273
B 197G	0·774	0·752

identical mixtures containing 400 Lf units of toxin and 400 units of antitoxin in volumes of 10 ml. were set up. After 4 days at 4° the floccules were spun down. In each case four of the precipitates were washed twice with 6 ml. chilled saline, and the other four were washed four times with 10 ml. chilled saline. The N contents of the washed floccules were then determined. We do not think the figures show important differences between the N contents of floccules washed with 12 ml. saline and of those washed with 40 ml. saline. (Each figure is the mean of four determinations.)

Table 10. *Different 'slopes' obtained with AS and NA toxin preparations*

Toxin preparation		Lf/mg. N	'Slope' N associated with 1 Lf unit toxin (mg.)	'Intercept' N associated with 1 unit antitoxin (mg.)
AS	211 L	14·4	0·0021	0·00189
	Pf 13 D	273	0·0020	0·00146
NA	B 259 F	815	0·00039	0·00140
	B 144 B	1250	0·00040	0·00154
	B 163 A	1500	0·00045	0·00145

While the 'slopes' for AS toxin preparations generally did not differ appreciably from 0·0020 mg. N associated/unit toxin, we found that the 'slopes' for the purer NA toxin preparations were much lower. This is shown in Table 10, where it will be seen that even though the AS toxin preparation 211 L (which was precipitated once only with $(NH_4)_2SO_4$) was nearly 20 times as crude as the AS preparation Pf 13 D they both had the same 'slope'. Thus it seems that the impurities which can be removed by ammonium sulphate fractionation, and which represent the difference between the preparations 211 L and Pf 13 D, were not adsorbed on the toxin-antitoxin floccules. On the other hand, some of the impurities which were removed during the nucleic acid precipitation of the toxin appeared to be adsorbed on the floccules, as is evident from the difference of the 'slopes' for NA and AS preparations.

That this difference was due to adsorption of non-specific impurities is also suggested by the following experiment: A batch of filtrate from a profuse culture of *Cl. welchii*, type A, strain S 107, which had produced practically no α-toxin, happened to be available. It was subjected to the $(NH_4)_2SO_4$ fractionation outlined in Table 1 and the resultant product (which contained less than 5 Lf/ml.) was mixed with the NA preparation B 259 F, which contained 385 Lf/ml., 815 Lf/mg. N, and which gave a 'slope' of 0·00039 mg. N. The contaminated preparation now contained 40 Lf/mg. N, and on flocculation it gave a 'slope' of 0·00089 mg. N.

It is unlikely that the difference in the 'slopes' given by AS and NA preparations was due to a flocculating antigen other than α-toxin present in the AS preparations and not in the NA preparations. If this were the case one would expect the 'intercepts' for the NA preparations to be considerably lower than those of the AS preparations, since the antibody to the secondary antigen would not be precipitated in the flocculations with the NA preparations. In fact the 'intercepts' for AS and NA preparations are much the same. It is also worth recording that the AS preparation free from α-toxin used in the experiment just described did not precipitate with the antitoxin preparation used in this work.

At one time we entertained the suggestion that the change in 'slope' might be due to a part of the toxin molecule, amounting to one fifth of the whole and containing all the catalytic and antibody-combining centres, being split off during the nucleic acid purification. In such an event an approximately 1·7-fold change in the diffusion coefficient could be expected. We measured the diffusion coefficients of the toxin diffusing from AS and NA preparations and found a difference which we do not consider significant. The values of D_{20} for AS and NA preparations respectively were 0·059 and 0·067 cm.^{2}/day. While we were measuring the diffusion coefficient of the toxin in the AS preparations we found that the pigment in these preparations also diffused at a uniform rate to give a value of $D_{20} = 0{\cdot}090$ cm.^{2}/day.

DISCUSSION

The Danysz phenomenon results from the fact that 1 unit of toxin can combine with more than 1 unit of antitoxin, and from the fact that the complex TA_x (where $x > 1$) thus formed reacts slowly (whether or not it is in solution) with toxin to form TA. These two facts explain the phenomenon completely, and we do not see why it is more difficult to understand when the toxin-antitoxin complexes do not precipitate, and why Pappenheimer & Robinson (1937) should have found it necessary to introduce further assumptions in order to explain it. The fact that diphtheria toxin and α-toxin can also form slow reacting complexes of the type T_yA (Pf 13 D, Table 1, and B 202 J, Table 3, appear to form T_2A) suggests that what might be called a 'converse' Danysz phenomenon can also be expected.

We have seen that by titration complexes of the type TA_x to T_yA (where x and y both > 1) have been detected, and that the limits of x and y vary with different batches of toxin. It appears that the toxin and antitoxin in complexes of this type (i.e. those in the vertically hatched areas in Fig. 1) are involved in such a way that the reactant in excess of the ratio TA is not completely free to react with its immunological opposite and consequently it reacts slowly.

in complexes in which the values of x and y are greater than those observed in the titrations (i.e. complexes in the horizontally hatched areas in Fig. 1) the additional reactant is not so closely involved and reacts rapidly with its immunological opposite to form TA_x or T_yA as the case may be. Since the limits of x and y vary with different batches of toxin it seems that the way in which the toxin and antitoxin are bound together must vary in some way from batch to batch.

The toxin-antitoxin reaction takes place in two stages (see Boyd, 1943). The first stage consists of the union of toxin and antitoxin to form a soluble complex,

$$xT + yA \xrightarrow{\text{fast}} T_xA_y;$$

the second stage (which does not necessarily always follow) consists of the aggregation of the complex to form an insoluble precipitate,

$$T_xA_y \xrightarrow{\text{slow}} (T_xA_y).$$

We suggest that the reason why different batches of α-toxin appear to give different 'intercepts' might be that, although the first stage of the reaction is quantitative (since no free toxin or antitoxin is found in the supernatants), the second stage may not be quantitative and the degree to which it proceeds varies from batch to batch. Pope & Healey (1939) have found that diphtheria toxin-antitoxin floccules cannot always be centrifuged down quantitatively. With some samples of unrefined antitoxin as much as 50 % of the complex remained in the supernatant after prolonged centrifuging; the effect was much less marked with pepsin-refined antitoxin. They also found similar losses on washing the floccules with saline. Our floccules did not show a tendency to remain suspended after washing (Tables 6 and 9).

It seems reasonably clear, in the case of *Cl. welchii* α-toxin at any rate, that impurities from the toxin preparations can be adsorbed on the toxin-antitoxin floccules, and that such impurities can account for at least 30–45 % of the N in the floccules. Apparently no impurities were adsorbed on the diphtheria toxin-antitoxin floccules observed by Pappenheimer & Robinson (1937) since the 'slope' they found agrees exactly with the N content of a unit of pure toxin (Pappenheimer, 1937). Pope & Healey (1939), however, drew attention to the adsorption of pigments and lipids on diphtheria toxin-antitoxin floccules, and we found that the *Cl. welchii* α-toxin-antitoxin floccules tended to be pigmented, even after washing. Moloney & Hennessey (1942) found a 'probable value' of 0·0004 mg. for the 'slope' of tetanus toxoid; Pillemer, Wittler & Grossberg (1946) isolated pure tetanus toxin containing 0·00025–0·000286 mg. N/Lf. Hottle & Pappenheimer (1941), working with two partially purified toxin preparations, found a 'slope' of 0·00023 mg. N associated/Lf unit of scarlet fever toxin. There is no proof that impurities were not adsorbed on their floccules, and in view of our experience we think that such proof should always be provided. In the case of our toxin preparations it seems that there are at least two kinds of impurities. The first kind of impurity is removable by ammonium sulphate fractionation and is not adsorbed on toxin-antitoxin floccules (compare preparations 211 L and Pf 13 D, Table 10); the second kind of impurity is only removed when the toxin is precipitated with nucleic acid, and it is adsorbed on the floccules (compare AS and NA preparations, Table 10). Apparently this impurity was not adsorbed from preparation B 197 G (Table 8) to the same extent as it was from other AS preparations. If the 'slopes' for the NA preparations represent the true value for the mg. N/Lf toxin, then the NA toxins are about 50 % pure, since the 'slopes' are about 0·0004 mg. N/Lf and the preparations contain about 0·0008 mg. N/Lf; but we have no proof that the floccules from these preparations also do not adsorb impurities and therefore we can only say that the preparations are not more than 50 % pure.

We have no means yet of knowing whether the adsorbable impurities are derived from the culture medium or from the organisms. The danger that such impurities may exist in any culture filtrate leads us to believe that only pure toxins and antitoxins can be used in quantitative flocculation studies. Although Pappenheimer & Robinson (1937) did not have trouble with adsorbable impurities in their diphtheria toxin preparations, such trouble might arise with other preparations of diphtheria toxin or of any other toxin. For this reason we do not think it safe to calculate the potency of impure toxin and antitoxin preparation from one (or more) nitrogen determination at the flocculation point as suggested by Pappenheimer & Robinson. We fear that in this respect we are on the side of the 'brass hats' (Heidelberger, 1946).

SUMMARY

1. Methods are given by which the α-toxin of *Cl. welchii* was routinely purified 50-fold by ammonium sulphate fractionation. By nucleic acid precipitation a further fivefold purification could be achieved. The final product was not more than 50 % pure.

2. The α-toxin was capable of combining with antitoxin to form, on the average, complexes containing toxin and antitoxin in ratios varying from T_2A_3 to T_6A_5 (where T and A represent Lf units of toxin and units of antitoxin respectively). These complexes, whether or not they were in solution, reacted slowly with toxin or antitoxin. With two batches of toxin, complexes of average composition TA_2 and T_2A have been detected.

3. Results obtained suggest that the formation of insoluble aggregates from soluble toxin-antitoxin complexes in the equivalence zone may not always be quantitative.

4. Impurities are adsorbed on the α-toxin-antitoxin floccules. The implications of this finding are discussed.

We are grateful to Miss A. G. Kennedy for many nitrogen determinations, to Miss Beryl Sievwright for much technical assistance, to Mr A. T. Glenny, F.R.S., and Mr H. Proom for the benefit of valuable criticism and stimulating discussion, and to Dr J. W. Trevan, F.R.S., for sympathetic encouragement. One of us (W.E.v.H.) also wishes to acknowledge the help given to him in Cambridge by Mrs E. E. Bacon.

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The Biochemistry of the Gas Gangrene Toxins

5. THE κ-TOXIN (COLLAGENASE) OF *CLOSTRIDIUM WELCHII*

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Maschmann (1937) described an enzyme in young culture filtrates of *Clostridium welchii* (*B. perfringens*) which 'could attack gelatin extensively, but not ovalbumin, casein and fibrin'; he gave details for its partial purification (Maschmann, 1938*a*) and suggested the name 'collagenase' for it (Maschmann, 1938*b*), but later (Maschmann, 1938*c*) withdrew the suggestion without giving reasons.

The possible importance of a collagenase in gas gangrene was discussed by Maschmann (1937, 1938*a*, *b*) and the subject has been reopened recently by Macfarlane & MacLennan (1945). They observed in cases of clinical gas gangrene in man, and in rabbit muscle injected *in vivo* with *Cl. welchii* culture filtrates, that the trypsin-insoluble collagen framework of the muscle was disintegrated leaving the muscle fibres intact. Teased collagen from human tendon was lysed by *Cl. welchii* culture filtrate; these findings were confirmed by Robb-Smith (1945). Macfarlane & MacLennan suggested that *Cl. welchii* collagenase might be responsible for the liberation from the tissues of the host of a non-specific toxic substance(s) which might play an important part in gas gangrene toxaemia. Evans (1945) found that the anti-collagenase titre of *Cl. welchii* type A antisera was not correlated with the protection of guinea pigs against experimentally produced gas gangrene by the sera. At the suggestion of Drs Macfarlane and MacLennan we undertook the work to be described in this paper.

Oakley, Warrack & van Heyningen (1946) showed by titration with a large number of *Cl. welchii* antisera that the muscle-disintegrating substance present in *Cl. welchii* type A filtrates was distinct from α-toxin, θ-toxin and hyaluronidase, and that it was antigenically indistinguishable from the substance in those filtrates which attacks collagen in the form of collagen 'paper', hide powder and 'azocoll'. They proposed that the antigen concerned should be called κ-toxin.

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We have succeeded in obtaining preparations of this collagenase free from other toxins of *Cl. welchii* and in a highly purified condition. In addition to its possible clinical interest, this collagenase is remarkable for its high substrate specificity, since it appears to attack only collagen and gelatin.

METHODS

Estimation of collagenase

The antibody-combining power, or antigen content, of collagenase preparations (expressed as κ units/ml.) was measured by the method of Oakley *et al.* (1946).

The enzymic activity (expressed as Q units/ml.) was determined by measuring the amount of dye liberated by the enzyme, under standard conditions, from hide powder to which R acid has been coupled ('azocoll', see Oakley *et al.* 1946). The Q unit is defined as that amount of enzyme which, under the conditions outlined below, will liberate twice as much dye from azocoll as is liberated in a blank.

Preliminary experiments with a photoelectric absorptiometer showed that the amount of dye liberated when a given amount of azocoll was shaken in 10 ml. buffer solution was sufficiently constant to be used as a colorimetric standard. The standard conditions chosen were as follows: 75 mg. of a given batch of 120-mesh azocoll were shaken for 1 hr. at 37° with 10 ml. buffer (equal vol. of M/15 phosphate buffer pH 7·4 and 1% NaCl). The reaction was stopped by filtering off undissolved azocoll. The amount of dye liberated, when enzyme solutions were incubated under the same conditions, was found to be a linear function of the enzyme concentration within the range 0·1–0·8 Q unit/ml., i.e. between the colorimetric ratios standard/unknown of 2–9, where the standard colour was that obtained in the blank as described above.

Since the amount of dye liberated varied with different batches of azocoll, it was important to use large batches. Most of our determinations were carried out with one batch of azocoll, and when a new batch was used it was necessary to determine its characteristics with a collagenase preparation the value of which had already been determined with the first batch. We cannot recommend the method since it cannot be truly standardized, and therefore we have not given it in detail. In our hands it was a rapid and fairly reliable means of following the progress of purification, which we regarded as an expedient to be discarded if a synthetic substrate were found.

Lecithinase (α-toxin) antigen was measured in Le units as described by van Heyningen (1948).

The efficiency of a procedure designed to separate collagenase from lecithinase was expressed by the ratio: Q units of collagenase/Le units of lecithinase in the product, to Q units/Le units in the original material.

Method of estimation of digestion of hide powder. Experiments were made with 120-mesh hide powder (specially prepared by Dr A. C. McDougall from hide powder supplied by Baird and Tatlock Ltd.) which had been washed with water and acetone and dried at 37°. Unless otherwise stated, the hide powder was incubated at 37° for 1 hr. in a total volume of 10 ml., in 25 ml. bottles which were agitated throughout that time. After incubation the mixtures were rapidly cooled to room temperature (within 0·5 min.) and filtered through Whatman no. 41 filter papers. Controls were run with corresponding heat-inactivated enzyme. Total N determinations were carried out on the filtrates. Results are usually shown as the difference between the total N of the filtrates when active and inactivated enzyme are used.

The action of the enzyme on gelatin. This was followed by determining the total N of the filtrate after the addition to the reaction mixture of 1/9th its volume of 5% (w/v) phosphotungstic acid (A.R.), in N-H_2SO_4.

Standard conditions obtained when 40 ml. of 0·5% (w/v) gelatin which had been incubated for 1 hr. at 37° with 5 ml. of enzyme solution were treated with 5 ml. precipitant. In experiments where gelatin solutions stronger than 0·5% were used, they were diluted at the end of the incubation, before adding the precipitant, so that the final concentrations were the same as for 0·5% gelatin. After thorough shaking, and standing for 5 min. the precipitates were filtered off on Whatman no. 3 papers. Heat-inactivated enzyme controls were treated in the same way. Results are shown as the difference between the total N of the filtrates when active and heat-inactivated enzyme preparations are used.

Other estimations. θ-Toxin by the method described by van Heyningen (1948); total nitrogen by micro-Kjeldahl procedure; NH_3-N by distillation in a Markham still (Markham, 1942) with 10 ml. of the phosphate buffer recommended by Sen (1942); 'tyrosine' by the method of Anson (1938); pH values were measured electrometrically with a glass electrode.

EXPERIMENTAL AND RESULTS

Purification of collagenase

The medium used in most of the work was a nutrient broth (4–5 g. total N/l.) containing 1% peptone, cooked meat and chalk. It was chosen, in preference to the medium described by van Heyningen (1948), because the resultant crude filtrate contains little if any θ-toxin. The organism was *Cl. welchii* type A, strain S 107.

Stage A. The culture filtrate was harvested after 5 hr. growth at 37°, the pH adjusted to 6·8, and $(NH_4)_2SO_4$ stirred in using 60 kg. $(NH_4)_2SO_4$/100 l. culture filtrate. After standing overnight the scum which floated on the surface was collected and dissolved in water. This solution will be referred to as the starting material.

The further stages in the purification were as follows:

Stage B. Adsorption on calcium phosphate followed by elution with 10% (w/v) $(NH_4)_2SO_4$.

Stage C. Charcoal treatment.

Stage D. Fractional precipitation with $(NH_4)_2SO_4$.

An outline of the procedure finally adopted may now be given.

Starting material from stage A was diluted to a collagenase concentration of 12–15 *Q* units/ml. and $(NH_4)_2SO_4$ added to a final concentration of 3·75% (w/v). A calcium phosphate precipitate was formed in the solution by adding 80 ml. M-$CaCl_2$ and 200 ml.

0·5M-phosphate buffer (pH 6·8) to each litre of diluted starting material. These additions were made in small portions (using volumes in proportion to total) with vigorous mechanical stirring. After 30–45 min. the precipitate was filtered off by suction on a Büchner funnel, washed with water (without loss of enzyme), and eluted by grinding thoroughly in a mortar with 10% (w/v) $(NH_4)_2SO_4$, 250 ml. being used for the precipitate from every litre of diluted starting material. The precipitate was left with the eluent for several hours or overnight at 4°, before filtering. The precipitate was usually eluted again with half the previous volume of 10% $(NH_4)_2SO_4$ and the two eluates combined (Eluate I).

Eluate I was stirred for 3–5 min. with an amount of Farnell's L.S. charcoal found to be suitable in a trial experiment (usually about 1%), and filtered. The filtrate was then dialyzed overnight against running tap water. Sufficient solid $(NH_4)_2SO_4$ was then added to the dialysate to bring the concentration to 3·75% (w/v) and the adsorption and elution with calcium phosphate repeated as before, to give Eluate II.

Eluate II was then dialyzed against running tap water (if it had retained too much colour it was first subjected to a second charcoal treatment as before). The dialysate was cooled in an ice-water bath and 2 vol. ice-cold 51% (w/v) $(NH_4)_2SO_4$ solution added slowly with stirring. After standing overnight at 4° the flocculent precipitate was collected by filtration, with the aid of a little 'Hyflo Supercel' (Johns-Manville Ltd.). The solid on the filter was extracted several times with small volumes of borate buffer or distilled water. The extracts were usually dialyzed against several changes of borate buffer (pH 7·4) in which the enzyme is fairly stable. A typical preparation is shown in Table 1.

Table 1. *Stages and yields in the purification of collagenase*

Stage	Description	Volume (l.)	Q* units/ ml.	κ* units/ ml.	Le* units/ ml.	Stage yield of collagenase (%)		Overall yield of collagenase (%)		Non-dialyzable nitrogen (NDN) (mg./ ml.)	κ units/ mg. NDN	κ/Le
						Calc. from Q-content	Calc. from κ-content	Calc. from Q-content	Calc. from κ-content			
A	Crude filtrate	180	2·1	3·2	3·4	—	—	—	—	0·283	11·3	0·94
	Pptd. with $(NH_4)_2SO_4$ (60 kg./100 l.). Scum dissolved in H_2O	21	11·6	29	22·8	64	106	64	106	1·19	24·4	1·27
B and C	First calcium phosphate treatment, giving Eluate I	7·87	23·1	44·6	5·8	75	57	48	61	0·12	374	7·7
	Treatment with charcoal (1·5% w/v)	7·87	19·5	39·2	3·8	85	88	41	54	0·051	770	10·3
	Second calcium phosphate treatment, giving Eluate II	3·9	23·3	62·5	—	59	79	24	42	0·046	1360	—
	Treatment with charcoal (0·8%)	3·78	17·7	38·5	—	74	60	18	25	0·035	1100	—
	Dialyzed overnight	5·08	12·8	29	<½	97	101	17	26	—	—	—
D	Fractional precipitation with $(NH_4)_2SO_4$. Solution of ppt. dialyzed against borate buffer (pH 7·4)	0·084	604	1230	5·5	78	70	13	18	0·590	2100	224

* Q = collagenase enzyme units; κ = collagenase antigen units; Le = lecithinase antigen units.

Determination of optimum conditions for the various stages of the process

(i) *Adsorption on calcium phosphate* (*stage B*)

Adsorption of collagenase on calcium phosphate provided the best means of separating it from lecithinase, but the conditions which give the maximum separation consistent with good yields of collagenase are dependent on several factors as outlined below.

Formation of the calcium phosphate precipitate. The precipitate was formed *in situ*, as it was found that not only did aged calcium phosphate give much poorer yields of collagenase, but the separation from lecithinase was also much less efficient.

pH *of the buffer used in the precipitation.* 50 ml. starting material (28 Q units/ml.; 40 Le units/ml.; 3·2% $(NH_4)_2SO_4$) at pH 6·3, were treated with 4 ml. M-$CaCl_2$ and 10 ml. 0·5M-phosphate buffer at a range of pH values (see Fig. 1). At

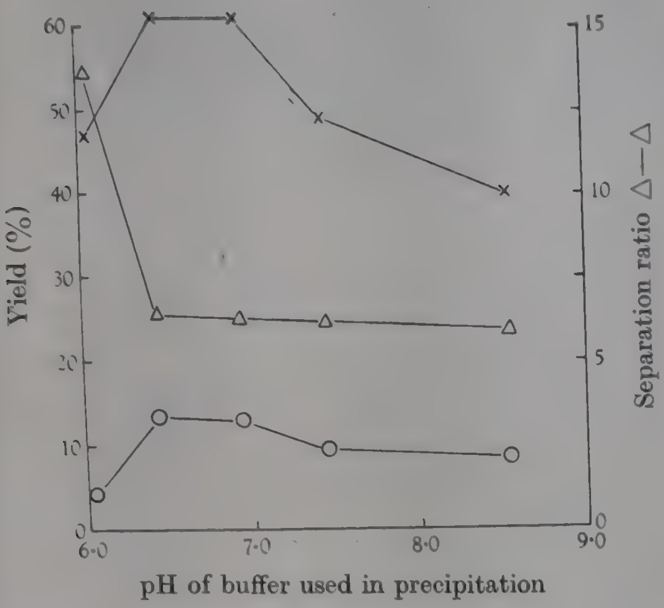


Fig. 1. The variation of yield of enzyme, and of efficiency of separation of collagenase from lecithinase, with the pH of the phosphate buffer used in the precipitation of calcium phosphate. The efficiency of separation of collagenase from lecithinase is measured by the separation ratio, i.e. the ratio Q units/Le units in the product to Q units/Le units in the starting material. Yield of collagenase enzyme in eluates ×—×. Yield of lecithinase antigen in eluates ○—○.

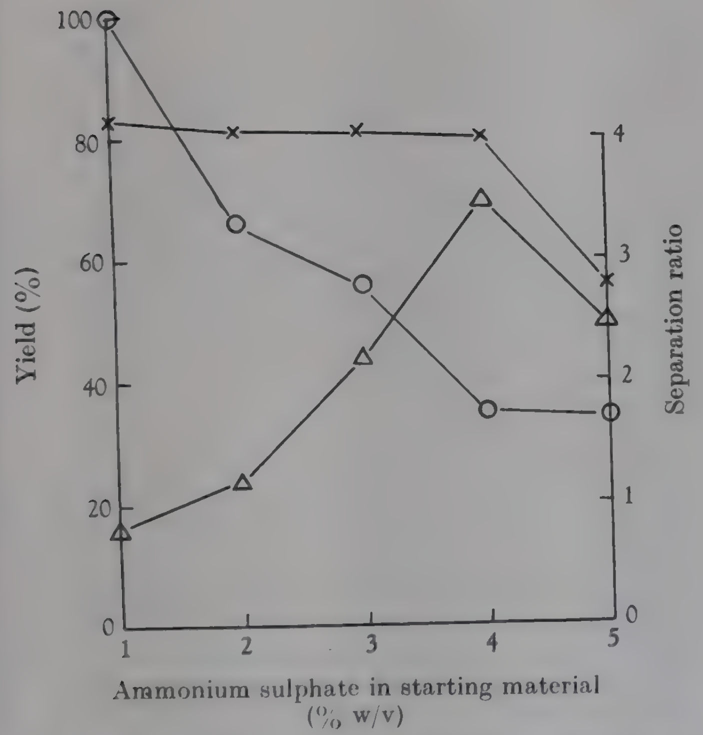


Fig. 2. The variation in yield of enzyme, and efficiency of separation of collagenase from lecithinase, with the concentration of ammonium sulphate in the starting material in the first calcium phosphate treatment. Separation ratio values and graphical symbols have the same significance as in Fig. 1.

pH 5·4 and below, no precipitate was formed. The precipitates were eluted with 50 ml. portions of 10% $(NH_4)_2SO_4$, and the eluates tested for collagenase and lecithinase content. Fig. 1 shows that the optimum pH appears to be 6·5–7·0, and in all further work 0·5 M-phosphate buffer at pH 6·8 was used. Variation of the pH of the starting material from pH 6·3 to 6·8 did not affect the result.

The concentration of $(NH_4)_2SO_4$ *in the starting material.* To 20 ml. portions of the enzyme preparations (*a*) or (*b*) (see below) were added varying amounts of $(NH_4)_2SO_4$ and water to 25 ml., 2 ml. M-$CaCl_2$ and 5 ml. 0·5 M phosphate (pH 6·8). Precipitates were eluted with 20 ml. 10% $(NH_4)_2SO_4$ and the eluates tested. Two preparations are described. Enzyme preparation (*a*): diluted starting material (11·5 Q units/ml.; 23·3 Le units/ml.; Q/Le = 0·49); see Fig. 2. Enzyme preparation (*b*): dialyzed eluate from a calcium phosphate treatment (Eluate I; 79 Q units/ml., 27 Le units/ml., Q/Le = 2·9); see Fig. 3.

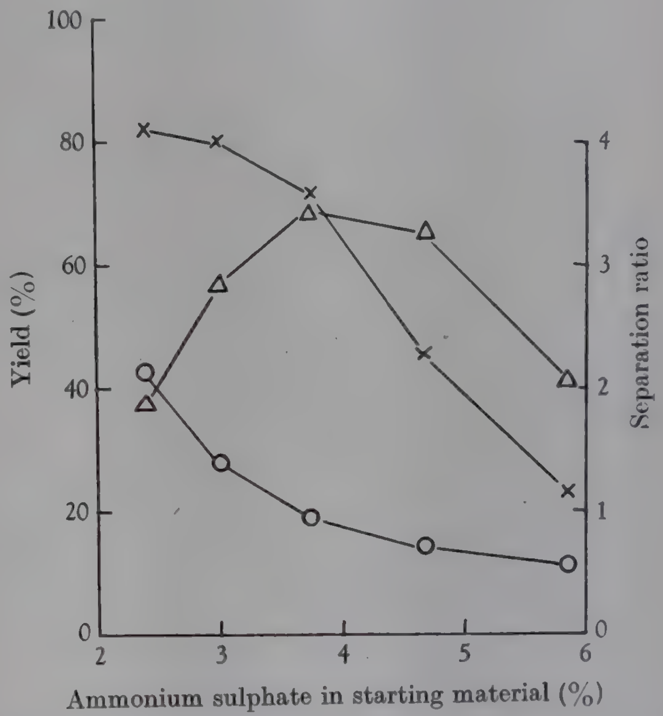


Fig. 3. The variation of yield of enzyme, and efficiency of separation of collagenase from lecithinase, with the concentration of ammonium sulphate in the starting material in the second calcium phosphate treatment. Separation ratio values and graphical symbols have the same significance as in Fig. 1.

The curves show that although the details differ for the two preparations, the optimum conditions are similar, and in all further work the concentration of $(NH_4)_2SO_4$ was adjusted to 3·75% (w/v) before the calcium phosphate treatment.

The concentration of enzyme in the starting material. Varying amounts of the enzyme preparations (*c*), (*d*) and (*e*) (see below) were diluted to the same volume, the concentration of $(NH_4)_2SO_4$ adjusted to 3·75% (w/v), and a calcium phosphate adsorption and elution carried out as usual. Enzyme preparation (*c*) was starting material derived from van Heyningen's medium (van Heyningen, 1948) (72 Q units/ml., 47 Le units/ml., Q/Le = 1·5) (see Fig. 4). Enzyme preparation (*d*) was starting material derived from meat-broth medium (50 Q units/ml., 178 κ units/ml., 120 Le units/ml., Q/Le = 0·42) (see Fig. 5). Enzyme preparation (e) was a dialyzed eluate from a first calcium phosphate treatment (Eluate I) (75 Q units/ml., 250 κ units/ml., 22 Le units/ml., Q/Le = 3·4) (see Fig. 6).

The curves in Figs. 4 and 5 show that, for the first treatment with calcium phosphate, the recovery of collagenase and separation from lecithinase were both improved as the initial concentration of enzyme was decreased, and that despite differences in the starting materials of preparations (c) and (d) (see Q/Le ratio), the results were qualitatively similar. In order to minimize the inconvenience of working with large volumes, we compromised by diluting the starting material to a collagenase concentration of 12–15 Q units/ml. in all our subsequent routine preparations.

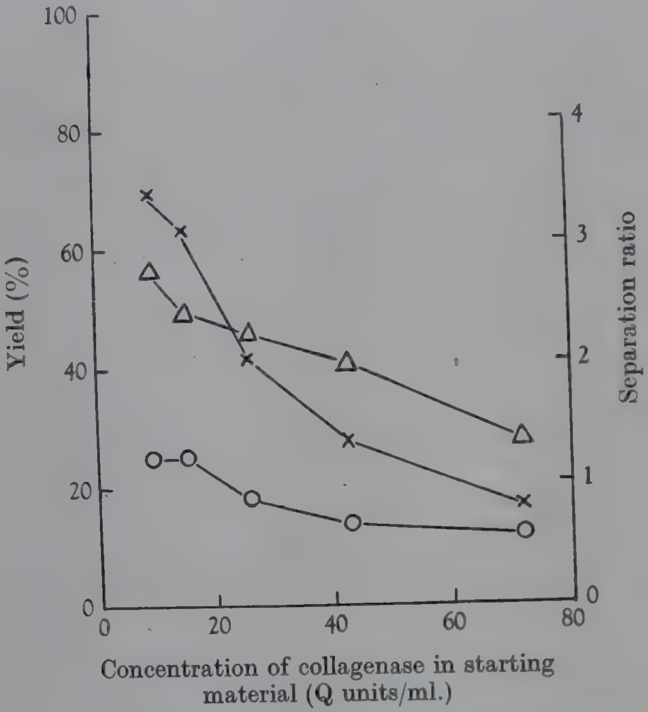


Fig. 4. The variation of yield of enzyme, and separation of collagenase from lecithinase, with the concentration of collagenase in starting material derived from van Heyningen's medium in the first calcium phosphate treatment. Separation ratio values and graphical symbols have the same significance as in Fig. 1.

Fig. 6 shows that for the second treatment with calcium phosphate best yields and separation are again associated with low initial enzyme concentrations, but this is much less marked than for the first calcium phosphate treatment. In practice second calcium phosphate treatments were always carried out without dilution.

(ii) *Charcoal treatment* (*stage C*)

Much pigment is removed in the calcium phosphate treatments, but sufficient remains to be troublesome in the final concentrates. Farnell's L.S. charcoal will remove most of the pigment and allow twofold purification of collagenase with 80–100 % yield. The charcoal treatment also has the advantage that lecithinase is adsorbed to a relatively greater extent than collagenase.

The eluate (containing 10 % $(NH_4)_2SO_4$) from either a first or second calcium phosphate treatment is stirred for 3–5 min. with an appropriate amount of charcoal as determined by previous experiment (usually about 1 %), and then filtered.

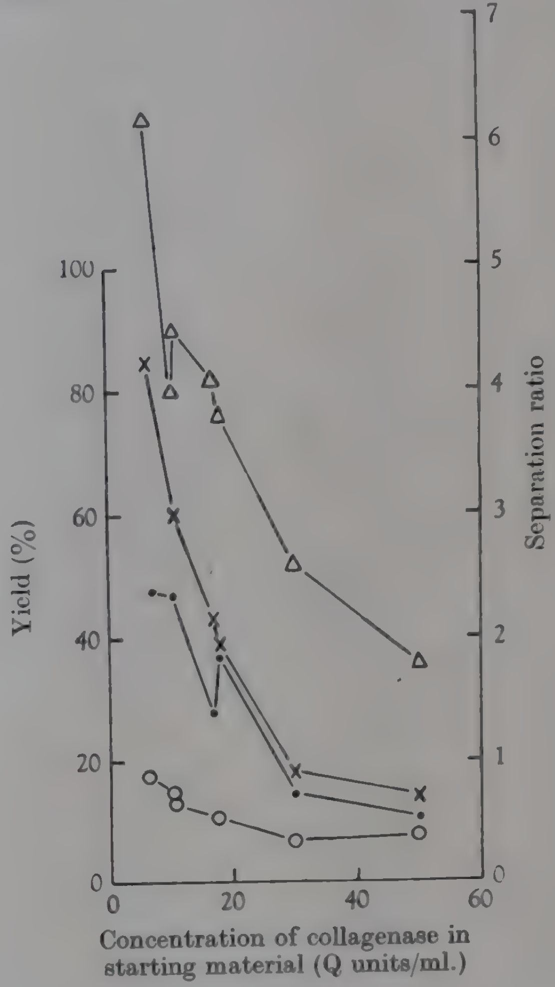


Fig. 5. The variation of yield of enzyme, and separation of collagenase from lecithinase, in starting material derived from meat broth medium in the first calcium phosphate treatment. Separation ratio values have the same significance as in Fig. 1. Yield of collagenase enzyme ×—×. Yield of collagenase antigen •—•. Yield of lecithinase antigen o—o.

Adsorption on hide powder

The enzyme can be adsorbed on hide powder and eluted from it, as shown by the following example:

75 g. of 60-mesh hide powder were added to 8·5 l. of dialyzed starting material (containing 49 Q units/ml.; 48 Le units/ml.), stirred for 1 hr., filtered, washed with 1 l. of water and eluted twice by stirring for 1 hr. with 500 ml. of buffer (M/30-phosphate, pH 7·4, containing 0·5 % NaCl (w/v)). The eluates were collected by filtration, the hide powder returned to the original supernatant and the process repeated twice. The combined eluates (2·9 l., containing 43 Q units/ml., and 0·5–1 Le unit/ml.) contained the collagenase in 34 % yield, and less than 1 % of the original lecithinase.

The use of hide powder for routine purification was, however, abandoned because of difficulties of filtration on a large scale, the partial solution of hide powder and its cost.

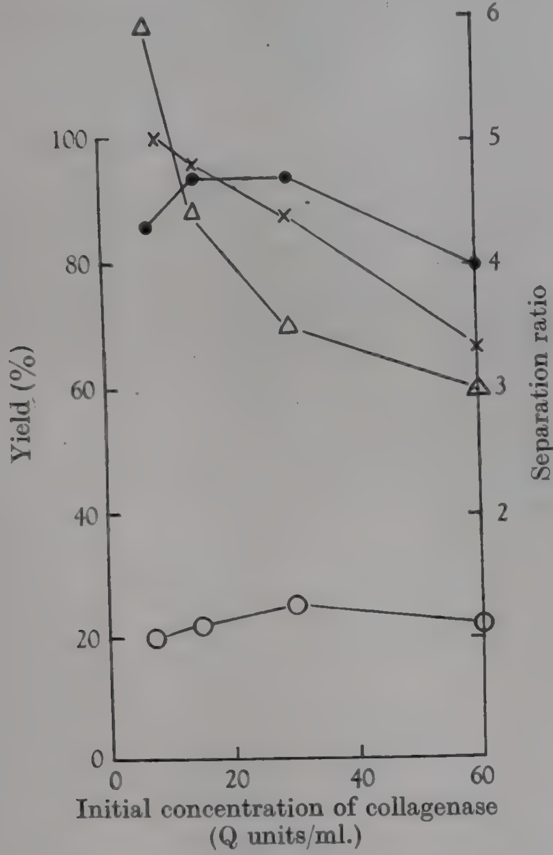


Fig. 6. The variation of yield of enzyme, and separation of collagenase from lecithinase, with the initial concentration of collagenase in a second treatment with calcium phosphate. Separation ratio values and graphical symbols have the same significance as in Fig. 5.

Properties of the purified enzyme

The purest preparation so far obtained (excluding material which has been subject to dialysis at pH 10·2—see below) contained 2600 Q units of collagenase/ml., 5800 κ-combining units of collagenase/ml., 33 Le units of lecithinase/ml., ≯ 10 units of θ-toxin/ml., 2·27 mg. N/ml., i.e. κ units/mg. N = 2550. (Preparations containing 12,000 κ units/mg. N have been obtained after dialysis at pH 10·2.) The hyaluronidase activity of the preparation has not been tested. The enzyme acts optimally over a range of pH 6·0–7·5, is stable in borate buffers at alkaline and neutral pH, but is relatively unstable at acid pH. (A preparation kept at pH 4·0 for 4 hr. at 4° and then restored to pH 6·8 had lost nearly 40 % of its activity.) Pure preparations are not stable in phosphate buffers; e.g. equal volumes of a given preparation were dialyzed for 3 days at 4° against several changes of (*a*) Palitzsch borate buffer pH 8·0 (Clark, 1928) and (*b*) M/15-phosphate buffer pH 8·0 with respectively 90 and 47 % recovery of collagenase activity.

Dialysis of purified collagenase preparations against several changes of borate-Na_2CO_3 buffer pH 10·2–10·4 (Clark, 1928) resulted in a considerable reduction in the test dose against κ-antitoxin in azocoll tests, i.e. an increase in the κ-combining power units/ml., sometimes, but not always, accompanied by a corresponding increase in enzymic activity, i.e. rise in Q units/ml.

For example a preparation B 269 H (2320 Q units of collagenase/ml., 7140 κ-combining power units of collagenase/ml.) in equilibrium with borate buffer pH 7·4, was redialyzed to equilibrium against borate-Na_2CO_3 buffer pH 10·4; the dialysate was unchanged in volume and contained 4100 Q units of collagenase/ml., and 14,300 κ-combining power units of collagenase/ml., corresponding to an increase of about 75 % in the enzymic activity and 100 % in the combining power.

It is possible that the dialysis at pH 10·2–10·4 causes the formation of active enzyme from some inactive precursor which also has different immunological specificity.

Parallel electrophoretic investigations by Mr P. A. Charlwood are discussed in the addendum to this paper.

Purified enzyme preparations containing *c.* 20 Q units of collagenase/ml. are inactivated by heating at 80° for 5 min.

We have confirmed the observation of Maschmann (1938*a*) that collagenase is partially inactivated in 0·001 M-solutions of copper and mercury ions (the latter being most effective). Manganese, cobalt, and iron salts (after long contact with the enzyme) are all inhibitory to varying extents. The decrease in enzymic activity is not accompanied by any change in the combining power. Magnesium and zinc ions appear to be without effect on the enzymic activity.

Kinetic studies

pH *optimum.* Hide powder (850 mg.) was incubated 1 hr. at 37° with 10 ml. of a mixture of 10 ml. buffer and 2 ml. enzyme, using (*a*) veronal-acetate-HCl buffer (Michaelis, 1931); (*b*) M/15-phosphate buffer; (*c*) Palitzsch borate buffer (Clark, 1928). Determinations of total N were made on the filtrates. Very little variation in the extent of digestion of the hide powder was observed over the range pH 6·0–7·5 (in the case of borate buffer, the lowest pH used was 6·9). Similar results were obtained with gelatin.

Substrate concentration. Column D of Table 2 shows that at concentrations of hide powder higher than 750 mg./10 ml. there is very little further rise in filtrate nitrogen, and further work on the kinetics was done with 850 mg./10 ml. owing to the difficulty in working with higher concentrations. It can be seen from column E (Table 2) that the amount of hide powder dissolved in the controls (heat inactivated enzyme) is approximately a constant percentage of the total amount present over the range 250–1000 mg./10 ml.

Table 2. *The optimum concentration of substrate for the action of collagenase on hide powder*

(Final collagenase concentration 0·28 Q units/ml. Buffer: Palitzsch borate pH 7·4.)

	Nitrogen content of filtrate (mg. N/10 ml.)			
Hide powder concentration (mg./10 ml.) A	When active enzyme used B	When heat-inactivated enzyme used C	Nitrogen content of filtrate due to enzyme action (mg. N/10 ml.) (B − C) D	Hide powder dissolved in controls* (%) $\left(\frac{C}{A}\times\frac{10^4}{15{\cdot}4}\right)$ E
100	8·4	3·7	4·7	24·0
250	17·0	7·6	9·4	19·8
500	28·8	14·0	14·8	18·2
750	40·3	20·6	19·7	17·9
1000	49·4	29·4	20·0	19·1

* In a separate experiment it was found that the hide powder used contained 15·4% nitrogen.

Table 3. *The optimum concentration of substrate for the digestion of gelatin by hide powder*

	Mixture incubated			Nitrogen content of filtrate (mg. N/100 ml.)			
N (mg./ml.) in gelatin substrate solution A	Gelatin (ml.) B	Buffer* (ml.) C	Enzyme† (ml.) D	When active enzyme used E	When heat-inactivated enzyme used F	Due to enzyme action (E − F) G	Col. G recalculated to refer to 80 ml. gelatin (mg. N) H
5·57	10	1·0	0·25	3·27	0·37	2·90	23·2
2·73	20	2·0	0·50	5·55	0·29	5·26	21·0
1·37	40	4·0	1·00	7·09	0·42	6·67	13·3
0·69	80	8·0	2·00	8·50	0·53	7·97	8·0

* Palitzsch borate pH 7·2.
† Active enzyme contained 21 Q units of collagenase/ml.

In determining the substrate concentration optimum for gelatin, the mixtures, made up as shown in Table 3, were incubated 1 hr. at 37°. Then sufficient ice-cold buffer (Palitzsch borate pH 7·2 in which all the solutions had been prepared) was added to bring the final volume to 90 ml., followed by 10 ml. precipitant (5% (w/v) phosphotungstic acid in N-H_2SO_4). Owing to the difficulty of preparing standard solutions of gelatin, the concentrations of the gelatin solutions used are indicated in column A of Table 3 by their nitrogen content. The values of mg. N/100 ml. filtrate due to the enzyme activity are shown in column G, Table 3. Column H gives the calculated values for the total amount of nitrogen 'soluble' under the given conditions (i.e. when 1 vol. of precipitant is added to 9 vol. of a mixture containing *c.* 0·6 mg. N/ml.) which would be produced by 2 ml. of the enzyme solution used, acting on 80 ml. of gelatin of the concentration given in column A in a total volume of 90 ml. A final gelatin concentration during incubation corresponding to about 5 mg. gelatin N/ml. seems to be about optimal.

Activity curves. Hide powder: see Fig. 7, curve *A*. M/15-phosphate buffer (pH 6·5), 850 mg. hide powder/10 ml., and incubation for 1 hr. at 37° were used.

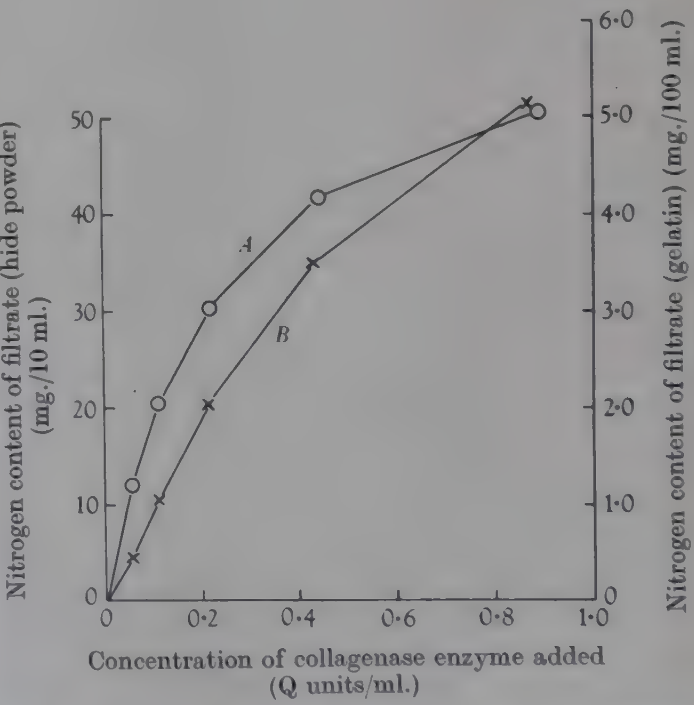


Fig. 7. Collagenase activity curves. *A*, with hide powder; *B*, with gelatin as substrates.

Gelatin: see Fig. 7, curve *B*. 10 ml. amounts of a solution of gelatin (5 mg. N/ml.) in borate buffer (pH 7·2) were incubated with amounts of enzyme as shown (total vol. during incubation, 11·25 ml.). After 1 hr. at 37°, 78·75 ml. ice-cold buffer was added, followed by 10 ml. precipitant; remainder of the procedure as usual. Controls: heat-inactivated enzyme treated in exactly the same way.

Interpretation of activity curves. Bergmann (1933) found that the enzymic digestion of gelatin sheets, the area of which remained constant during digestion, could be described by the formula: $x = kt\sqrt{E}$, where x = percentage digestion; k = constant; t = time; E = percentage enzyme concentration, and that the formula also held for the early part of the digestion, of fibrous collagen, i.e. these reactions followed the Schutz rule. He suggested that this could be explained by the assumption that the digestion of collagen fibres proceeds only from their ends. Fig. 8 shows that the digestion of hide powder and gelatin (in solution) by low concentrations of *Cl. welchii* collagenase in a given time also proceeds approximately according to a similar expression, $x = k\sqrt{E} - a$, where a is a constant, possibly due to partial inactivation of the enzyme during the course of the incubation, and that the kinetics of digestion of undissolved hide powder and dissolved gelatin are similar. Therefore we feel that Bergmann's suggestion that collagen fibres are digested only from the ends needs re-examination.

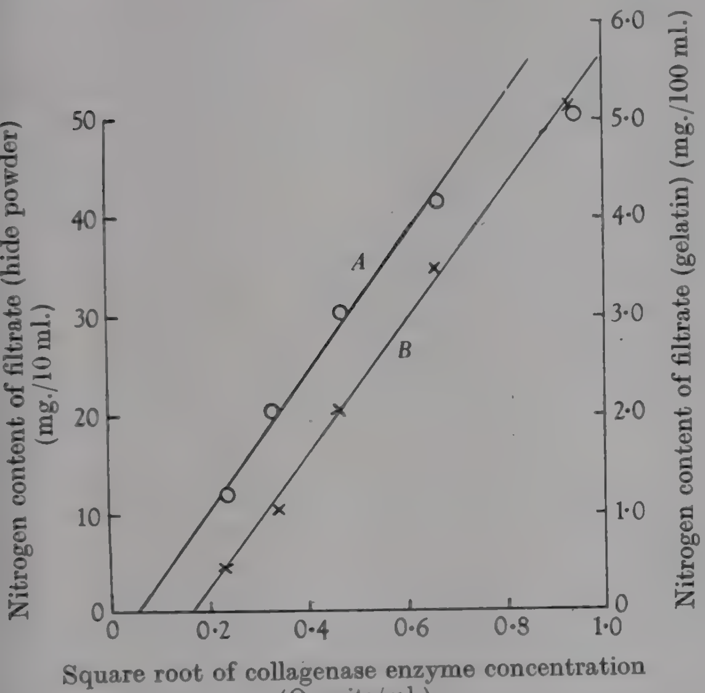


Fig. 8. Data from activity curves replotted to show variation in activity with the square root of the enzyme concentration. *A*, hide powder; *B*, gelatin.

Substrate specificity

Proteins other than collagen or gelatin. 5 ml. of substrate solution or suspension (containing about 2% protein) were incubated with 1 ml. enzyme solution for 1 hr. at 37°, then 10 ml. 0·3 M-trichloroacetic acid were added, and the solution filtered through Whatman no. 3 filter papers. Controls: 5 ml. substrate, 10 ml. 0·3 M-trichloroacetic acid added, then 1 ml. enzyme, filtered. The extent of digestion was estimated by either 'tyrosine' or total N determination on filtrates.

(*a*) Urea-denatured haemoglobin, prepared according to Anson (1938): little or no effect in the absence of cysteine, slight digestion in the presence of cysteine.

(*b*) Casein (British Drug Houses Ltd. L.W.S.): little or no effect.

(*c*) Normal horse serum: no effect.

(*d*) Urea-denatured normal horse serum: little or no effect.

(*e*) Heat-denatured horse serum albumin: no effect in the absence of cysteine; in presence of cysteine, about 10% of the substrate was digested. The experiment was repeated with a more highly purified preparation of collagenase, which showed no activity against heat-denatured horse serum albumin, even in the presence of cysteine and during 20 hr. incubation at 37°.

(*f*) Clupein sulphate: no action even during 20 hr. in the presence of cysteine.

The final collagenase concentration in experiments (*a*)–(*d*) was 16 Q units/ml., and in (*e*) was 7 Q units/ml. For comparison, it may be calculated from the data in Table 2, that collagenase in a final concentration of 0·28 Q units/ml. digests nearly 25% of the hide powder present (in 2·5% suspension) in 1 hr. at 37°.

Titration of groups liberated by the action of collagenase on gelatin. After incubation of gelatin with collagenase samples were titrated as follows:

(1) For amino nitrogen by the method of Pope & Stevens (1939).

(2) By the KOH-ethanol method (Grassmann & Heyde, 1929).

(3) By the HCl-acetone method (Linderstrøm-Lang, 1928).

In the control experiment a corresponding amount of heat-inactivated enzyme was used, the values were subtracted from those obtained where the active enzyme was used, and the result expressed in terms of m.equiv. of —NH_2 or —COOH formed by the enzyme in the whole mixture incubated (50 ml.). The conditions of the experiment were such that method (2) should measure the total acids formed, and method (3) the total —NH_2 groups formed. (For a critical study of these methods, see Richardson, 1934.) Method (1) provides a check on the others. The experiment was carried out on duplicate mixtures. The results (Table 4) were consistent with the splitting of a peptide bond.

Possible synthetic substrates. The following substances were found to be unaffected by collagenase: hippuramide, L-glycylproline, L-carbobenzyloxyprolylglycine, pyroglutamic acid, *O*-acetyl-L-hydroxyproline, *ON*-diacetyl-L-hydroxyproline.

Table 4. *The —NH_2 and —COOH groups liberated by the action of collagenase on gelatin*

(50 ml. gelatin solution (final concentration of gelatin N, 5·6 g./l.) with sodium chloride, 1% (w/v) incubated at 37° for 20 hr. with collagenase (1 Q unit/ml.) or the corresponding amount of heat-inactivated enzyme. All solutions adjusted to pH 7·0 before mixing. Toluene (2 drops) added to each mixture.)

Method of estimation	m.-equiv. of —NH_2 or —COOH formed
Pope & Stevens's (1939) copper method	1·90 (—NH_2)
HCl-acetone titration	2·08 (—NH_2)
KOH-ethanol titration	2·14 (—COOH)

Neutralization by antibody

It was shown by Oakley *et al.* (1946) that antisera produced in horses in response to immunization with culture filtrates of *Cl. welchii* type A generally contained antibody which inhibited the action of collagenase on collagen in the form of azocoll and collagen 'paper'. It was therefore to be expected that such antisera would neutralize the gelatin-splitting activity of our enzyme preparations. However, not only could inhibition not be demonstrated satisfactorily, but in some cases mixtures containing excess antiserum were apparently more active than when no antiserum was present.

Example: Varying amounts of antiserum R 8537 (from the same batch as the standard serum R 8531 of Oakley *et al.* (1946) and defined as containing 180 anti-κ units/ml.) were added to 1·0 ml. enzyme preparation (26 Q units/ml., 102 κ-combining units/ml.) diluted in sufficient borate buffer (pH 7·4) to make the final volume 7·5 ml. After standing 0·5 hr., 5 ml. amounts of the mixtures were incubated with 40 ml. gelatin solution (0·73 mg. N/ml.) for 1 hr. at 37°. 5 ml. of the usual precipitant were added and total N determinations were carried out on the filtrates. The controls consisted of a parallel series of mixtures, using heat-inactivated enzyme, which were incubated in exactly the same way.

The results are given in Table 5. All the mixtures were tested for activity against azocoll by the incubation overnight at 37° of 0·5 ml. of mixture with 1 ml. 'azocoll-manucol' suspension (Oakley *et al.* 1946). The results are shown in Table 5, column F. As expected, only the first mixture gave a strongly positive reaction (+ +), while all the others were completely negative. Mixtures 2 and 3 were undoubtedly over neutralized as far as activity against azocoll is concerned. The failure to neutralize the activity against gelatin might be due to one of several causes:

(*a*) Part, if not all, of the 'gelatinase' activity might be due to an immunologically distinct enzyme. If this were so, the 'gelatinase' would appear to be a very poor antigen, since several antisera having very varied contents of other antibodies have been shown to be ineffective in neutralizing this activity against gelatin.

(*b*) Dissociation of the complex formed by the enzyme with the homologous antibody. This appears an unlikely explanation in view of the fact that the final concentration of antiserum was over 50 anti-κ units/ml. and the incubation time only 1 hr., while the usual concentration in testing for combining power (κ-unitage) is less than 1 anti-κ unit/ml. and the time of incubation overnight.

(*c*) The anti-collagenase antibody may prevent gross particles of hide powder, but not the comparatively small molecules of gelatin, from reaching the catalytic centre of the enzyme. It has previously been observed that in some cases enzyme-anti-enzyme complexes are formed which retain all the activity of the uncombined enzyme, for example, the work of Campbell & Fourt (1939) on catalase.

None of the above explanations accounts for the rise in activity which has sometimes been observed in the presence of serum. That this is not due to any direct action of the serum on gelatin is shown by the controls (5 and 6, Table 5); the rise shown (over control 4) is probably due only to non-precipitable nitrogenous material in the added serum, and is in any case so small as to be negligible in the present discussion.

Table 5. *The effect of serum containing anti-collagenase antibody on the action of a collagenase preparation on gelatin*

Mixture no.	Added to 40 ml. gelatin: κ units of collagenase	Added to 40 ml. gelatin: Anti-κ units of serum	Nitrogen content of filtrate (mg. N/50 ml.)	Nitrogen content of filtrate produced by active enzyme (mg. N/50 ml.)	Activity of mixtures against azocoll
A	B	C	D	E	F
1	68	0	4·64	4·23	+ +
2	68	240	6·96	6·27	No detectable activity
3	68	360	8·29	7·39	"
4	<1/3	0	0·41	—	"
5	<1/3	240	0·69	—	"
6	<1/3	360	0·90	—	"

DISCUSSION

According to the theories of Bergmann and his colleagues (Bergmann & Fruton, 1941) the specificity of proteolytic enzymes is governed by the nature and arrangement of the amino-acid residues on either side of the peptide bond that is hydrolyzed. The arrangement of amino-acids to which a given enzyme is specific may not occur in certain proteins, in which case those proteins would not be attacked by the enzyme; but it is surprising that out of the number of proteins tested the appropriate conditions of specificity for the collagenase of *Cl. welchii* appears to be present only in collagen (and its derivative, gelatin). This protein differs from other proteins in its high content of proline and hydroxyproline, but the narrow specificity of collagenase does not appear to be connected with these amino-acids. The failure of the enzyme to hydrolyze glycylproline or carbobenzyloxyprolylglycine indicates that the enzyme is neither a prolidase (Bergmann & Fruton, 1937) nor a prolinase (Grassmann, Schoenebeck & Auerbach, 1932).

We were prepared to consider the possibility that collagenase is not a peptidase, but that it dissolves collagen and liquefies gelatin as a result of its catalyzing some reaction other than the hydrolysis of a peptide bond. For example, it is conceivable, as Dr A. Neuberger suggested to us, that collagen and gelatin might contain ester linkages between the —OH groups of hydroxyproline residues and those carboxyl groups of glutamic acid that are not involved in peptide bonds. Hydrolysis of such linkages by an esterase might result in the solution of collagen or the liquefaction of gelatin. For this reason we tested the action of our enzyme preparations on *O*-acetyl-L-hydroxyproline and on *ON*-diacetyl-L-hydroxyproline, but no hydrolysis resulted.

On the other hand, the enzyme does appear to catalyze the hydrolysis of peptide bonds since amino-acids are liberated by it from gelatin, as is evident from our results. (Maschmann (1937) estimated his gelatinase by titrating the liberated amino groups.) The production of free amino groups is very slow compared with the solution of collagen or the liquefaction of gelatin.

The increase in κ-combining units of collagenase antigen (i.e. the decrease in the κ test dose), which occurred when partially purified preparations of the enzyme were dialyzed against borate-Na_2CO_3 buffer (pH 10·2–10·4), was confirmed on several occasions. It was often accompanied by an increase in enzyme activity (as measured by a rise in Q units/ml.). The enhancement of the combining power and enzymic activity was retained on redialyzing to equilibrium against pH 7·4 borate buffer. It is possible that the dialysis at pH 10·2–10·4 causes the formation of collagenase from some inactive precursor, which also has different immunological specificity. The parallel electrophoretic investigations described by Mr P. A. Charlwood in the addendum to this paper lend some support to this view, since a comparison of the electrophoretic properties (at pH 7·4) of a preparation before and after an intermediate dialysis at pH 10·2 (in borate-Na_2CO_3) shows that this treatment has produced some irreversible change in the protein constitution of the mixture.

SUMMARY

1. The preparation, from culture filtrates of *Cl. welchii* type A, of a proteolytic enzyme, 'collagenase' (κ-toxin), which appears to attack only collagen and gelatin, is described. It has been obtained substantially free from the α- and θ-toxins of *Cl. welchii* and purified (with respect to non-dialyzable nitrogen) about 200-fold.

2. The partially purified enzyme is optimally active over the pH range 6·0–7·5, and is stable in solution in borate but not in phosphate buffers.

3. An increase in activity and antibody-combining power after dialysis against borate buffer at pH 10·2 was observed.

4. Sera neutralizing the activity of collagenase-containing filtrates against collagen do not inhibit their activity against gelatin.

5. The electrophoretic properties of partially purified preparations are discussed in an addendum.

We are grateful to Dr R. G. Macfarlane and Dr J. D. MacLennan for suggesting this problem. We also wish to thank Miss G. H. Warrack for many immunological tests, Miss A. G. Kennedy for many nitrogen and other determinations, Mr G. A. Spiby for much technical assistance, Mr J. C. Macsween and the staff of the toxin laboratories for the provision of the crude filtrates, Dr S. Wilkinson of the Wellcome Chemical Research Laboratories for synthesizing the various peptides, etc., mentioned and Dr A. C. McDougall of the Wellcome Chemical Works for the preparation of 120-mesh hide powder. We are grateful to Dr C. L. Oakley for many helpful discussions.

Addendum. Electrophoresis of Collagenase Preparations

By P. A. CHARLWOOD

Experimental. The electrophoresis of collagenase preparations was carried out in a Tiselius apparatus at +0·5°, the diagonal schlieren method being employed. Preparations were dialyzed against borate or borate-carbonate buffer at the required pH. The buffer was renewed every 12 hr., and equilibrium was considered to be attained when no difference could be detected between the refractive indices of fresh buffer and buffer which had been in contact with the sample for 12 hr. Potential gradients employed in the U-tube ranged from 2·7 to 3·1 V./cm.

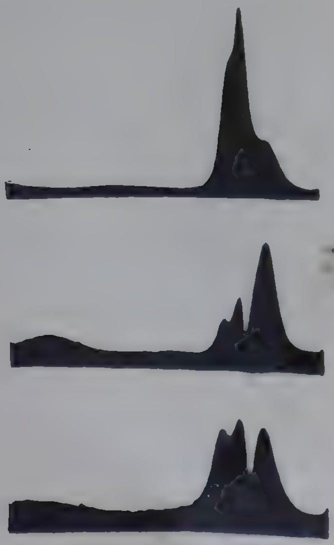
Table 6. *Collagenase preparations used in the electrophoretic experiments*

Sample	Q units/ml.	κ units/ml.	mg. N/ml.	κ units/mg. N	θ units/ml.	Le units/ml.
B 285B	3,200	7,000	3·22	2,180	<1	37·8
(B 286A)	(2,220)	(5,100)	—	—	—	—
B 286B	3,000	11,100	1·79	6,200	—	—
(B 287A)	(2,360)	(7,140)	—	—	—	—
B 287B	2,050	7,500	1·37	5,500	—	—

The collagenase preparation B 285B was dialyzed at pH 7·4 and a run carried out (see Fig. 9A). The material recovered from the U-tube (unavoidably diluted with buffer) was combined with the rest of B 285B, and filtered. The filtrate (B 286A) was dialyzed against buffer at pH 10·2; the dialysate (B 286B) was unchanged in volume and Table 6 shows that this treatment resulted in a large increase (over 200%) in the total κ units present. Preparation B 286B was submitted to electrophoresis (see Fig. 9B). Again the residues were collected (B 287A), dialyzed back to pH 7·4 (B 287B) without significant change in volume and Table 6 shows that there was no loss in κ-unitage during the return to pH 7·4. A final run was then carried out on preparation B 287B (see Fig. 9C).

Results of electrophoretic experiments. The electrophoretic pattern of B 285B (Fig. 9A) shows three peaks. The pattern of B 286B (Fig. 9B) also shows three peaks, but is considerably different, as the fastest-moving component now constitutes the major portion of the protein. The final material, B 287B (Fig. 9C) is clearly different from B 285B. These two, being at the same pH, should be almost identical if the sequence of pH changes had not affected the material present. The fastest-moving component in B 287B is present in much greater proportion than in B 285B.

The above series of changes in the curves correlates with the alteration in combining power. It would appear that the combining power is associated



	pH	Buffer	Potential gradient (V./cm.)	Time between starting current and exposure (min.)
A	7·4	Borate	3·10	335
B	10·2	Borate-carbonate	2·68	192
C	7·4	Borate	3·02	230

Fig. 9. Electrophoresis of collagenase. Anode (ascending) limb curves are shown. Migration is from left to right.

chiefly or wholly with the fast component. It would be of great interest to separate electrophoretically a pure specimen of this component and to ascertain whether or not this is the case. The chief difficulty lies in achieving a sufficiently good separation of pure material from the amounts of the preparations available.

Mobility determinations were made at pH 10·2 and 7·4. The results are shown in Table 7. They are uncorrected for buffer viscosity, and are also liable to contain errors caused by the inability to maintain a very steady current, owing to large fluctuations of the mains supply.

Table 7. *Mobilities of components present in collagenase preparations, expressed in cm./sec./V./cm.* $\times 10^{-5}$

	Component		
pH	Fast	Middle	Slow
10·2	−7·29	−5·96	−5·41
7·4	−5·25	−4·37	−3·76

The above results have been confirmed qualitatively by runs on other batches of collagenase preparations.

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The Colorimetric Determination of Stilboestrol and Dienoestrol

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A preliminary account has been given (Warren & Goulden, 1945) of investigations that have been carried out on methods proposed for chemical assay of synthetic oestrogens. Two methods were studied. One (Dingemanse, 1939) is based upon the development of a red colour by the oestrogen in the presence of antimony pentachloride; the other (Malpress, 1945) relies on the measurement of the yellow colour obtained by nitration of the oestrogen followed by neutralization. The antimony reaction proved to be very sensitive. By its use the instability of dienoestrol in solution was demonstrated. On the other hand, stilboestrol (4:4′-dihydroxy-αβ-diethylstilbene) appears to be quite stable in solution in a variety of solvents. It was subsequently found (Wilder Smith & Williams, 1945) that the oestrogenic activity of solutions of dienostrol shows a similar decrease with time. Hence, chromogenic activity towards antimony pentachloride and oestrogenic activity appear to run parallel. On the other hand, solutions of dienoestrol that show very low activity in the antimony pentachloride reaction continue to react strongly in Malpress's test.

In the present paper details of these experiments are given, together with the results of some attempts to apply the antimony reaction to the estimation of synthetic oestrogens in urine of subjects treated therapeutically with these substances.

EXPERIMENTAL

The antimony pentachloride reaction

In the original description of this reaction (Dingemanse, 1939), few details are given. Stilboestrol dissolved in chloroform (washed free from ethanol) was treated with a 50% (v/v) solution of antimony pentachloride in the same solvent. Difficulty was experienced in obtaining reproducible results by this simple technique, and a systematic study of the factors influencing colour development was undertaken.

Table 1. *Colour reactions of substituted stilbenes with antimony pentachloride*

Substance	Colour
Hexoestrol ($HO.C_6H_4.CHEt.CHEt.C_6H_4.OH$)	Transient yellow
Dienoestrol ($HO.C_6H_4.C(:CHMe).C(:CHMe).C_6H_4.OH$)	Red-brown
Stilboestrol ($HO.C_6H_4.CEt:CEt.C_6H_4.OH$)	Red
4:4′-Dihydroxy-αβ-dimethylstilbene	Red
4:4′-Dihydroxy-α-*n*-propylstilbene	Orange
4:4′-Dihydroxy-α-methyl-β-*n*-propylstilbene	Red
4:4′-Dihydroxy-α-ethyl-β-cetylstilbene	Red
4-Methoxy-αβ-diethylstilbene	Red-green
Stilbene	Yellow
α-Ethylstilbene	Brown
Triphenylethylene	Yellow-brown
α-(*p*-Hydroxyphenyl)stilbene	Brown-orange
Triphenylmethylethylene	Olive-green
4:4′-Diacetylstilbene	No colour
4:4′-Diacetyl-αβ-diethylstilbene	No colour

(*a*) *Specificity.* Many stilbene derivatives develop colour in the presence of antimony pentachloride dissolved in ethanol-free chloroform (small amounts of ethanol change, or destroy, the colour). In Table 1 the results of qualitative tests on a range of substituted stilbenes are shown. The presence of at least one ethylenic bond appears to be essential for chromogenic power (hexoestrol gives practically no colour), but the colour is modified by the presence of substituent groups in the molecule. In general, the presence of a hydroxyl group tends to deepen the colour towards the red end of the spectrum. Weight for weight, stilboestrol develops more colour than does dienoestrol. For quantitative measurement of stilboestrol and of dienoestrol (using a Spekker photoelectric absorptiometer) Ilford Spectrum Green filter no. 604 was found to be the most suitable.

(*b*) *Solvent.* The preliminary experiments were carried out in chloroform (B.P. grade) which had been thoroughly washed with water to remove the small quantity of ethanol normally incorporated. The chloroform was dried over calcium chloride and distilled before use. Good colour development was obtained on mixing a chloroform solution of antimony pentachloride with a chloroform solution of the oestrogen, but the colour faded rapidly. In order to overcome this difficulty other chlorinated solvents were tried. Carbon tetrachloride was unsuitable since, although a red colour developed rapidly, there was almost complete precipitation of a red compound. Trichloroethylene also had to be excluded since it reacts with antimony pentachloride and develops colour. Ethylene dichloride was finally chosen as being the most suitable of the solvents tried. Good colour development occurs and the colour is much more stable than in chloroform. An additional practical advantage is that a good commercial grade of this solvent can be used without preliminary purification. Previous washing with water or with alkali followed by drying and distillation effected no improvement in colour stability.

(*c*) *Concentration of antimony pentachloride.* The intensity of the final colour depends on the concentration of antimony pentachloride present. In order to establish the conditions for maximal colour development, 1 ml. of a solution of stilboestrol was treated with 9 ml. of solutions of antimony pentachloride of varying strengths. The results of these experiments are shown in Fig. 1. It will be seen that maximum colour is obtained when the final antimony pentachloride concentration lies between 1 and

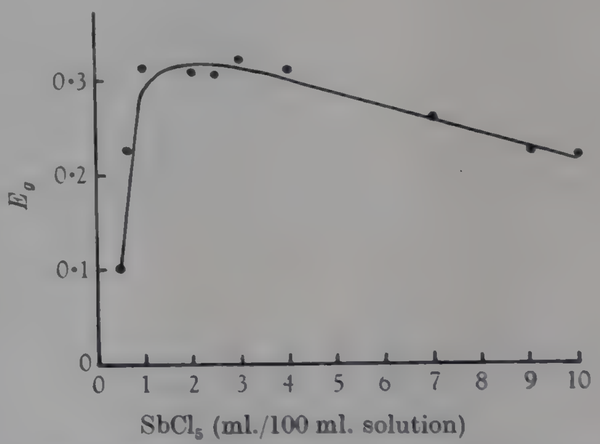

Fig. 1 Stilboestrol-$SbCl_5$ reaction. Influence of variation in concentration of $SbCl_5$ on the final intensity of colour. E_g is the extinction of the solution in green light.

4% (v/v). A reagent containing 3 ml. of antimony pentachloride dissolved in 100 ml. of ethylene dichloride was finally adopted as standard. For estimations of stilboestrol or dienoestrol, 9 ml. of this reagent were added to 1 ml. of a solution of the oestrogen in ethylene dichloride. Ordinary reagent quality antimony pentachloride (B.D.H.) was found quite satisfactory without any special purification, but the solution in ethylene dichloride employed as reagent should be freshly prepared immediately before use.

(*d*) *Time.* With dienoestrol, maximum colour development occurs immediately on mixing with the reagent. With stilboestrol the colour increases during the first 10 min. and then reaches a maximum. The influence of time on colour development is illustrated in Fig. 2. As a routine, colour measurements were made 15 min. after mixing the oestrogen with the reagent.

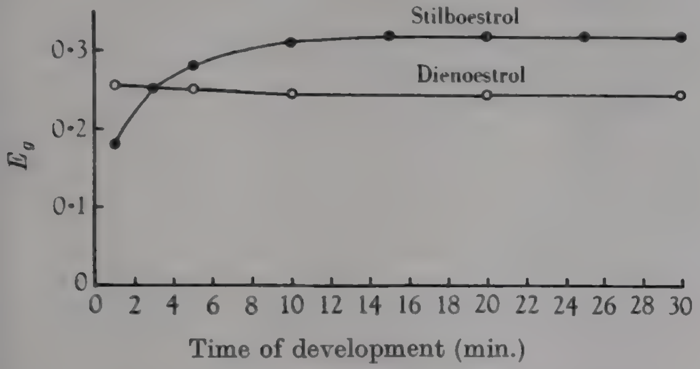

Fig. 2. Stilboestrol and dienoestrol colour reaction with $SbCl_5$. Effect of time of development on the final intensity of colour. E_g as in Fig. 1.

(*e*) *Temperature.* The rate of development of maximal colour varies with temperature. Although the temperature coefficient is not large it was found convenient to standardize the conditions by maintaining the solutions at 30° during the 15 min. period of colour development.

The Malpress nitro reaction

A comparison was made of this method (Malpress, 1945) with the antimony pentachloride method. A detailed account of the application of the method has been given by Malpress, and only points of major interest will be mentioned here.

(*a*) *Specificity.* The nitro reaction (a general one for phenols) is comparatively non-specific, both as regards substances giving the reaction and as regards colour developed.

(*b*) *Spectral absorption characteristics and sensitivity.* In Fig. 3 are shown approximate light absorptions (measured on a Hilger Spekker absorptiometer fitted with Ilford Spectrum filters) of oestrogens treated with antimony pentachloride and of dienoestrol treated according to Malpress's procedure. It will be seen that, whereas the antimony reagent develops a red colour with a definite maximum absorption in the green, the nitro reaction leads to an orange colour showing rather general absorption at the violet end of the spectrum.

An Ilford Spectrum blue-green (no. 603) filter gave the highest values and most sensitive slope to the calibration curve for the nitro reaction. When this filter is used the sensitivity of the nitro-reaction determination is about one twentieth that of estimations by the antimony reagent.

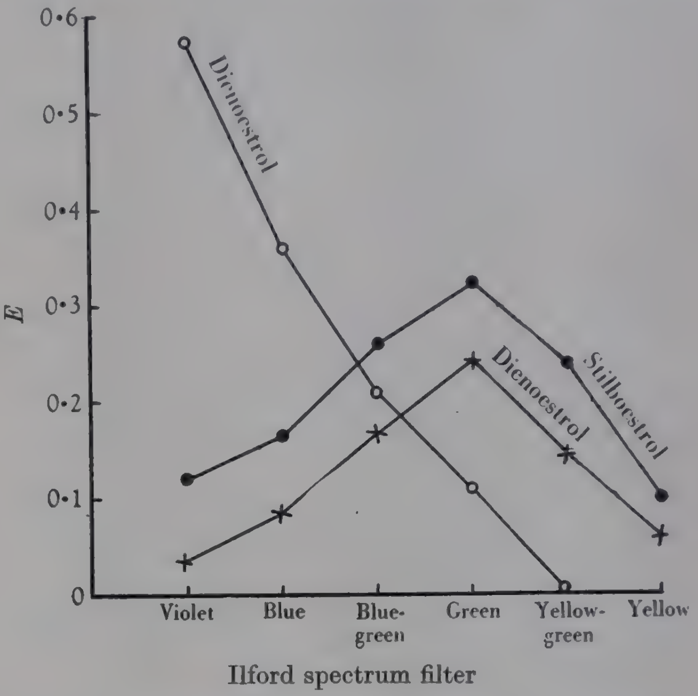

Fig. 3. Comparison of $SbCl_5$ reaction (stilboestrol and dienoestrol) and the nitro reaction (dienoestrol). Light absorption curves of the coloured products of these reactions. $SbCl_5$ reaction, oestrogen concentration 2 μg./ml. Nitro reaction, oestrogen concentration 20 μg./ml.

Application of the antimony pentachloride reaction to the determination of synthetic oestrogens in chloroform and in ethylene dichloride solutions

Since the ultimate objective of this work was the devising of a method for colorimetric determination of synthetic oestrogens in extracts prepared from human urine, the behaviour of these substances in solvents suitable for urine extraction (e.g. chloroform and ethylene dichloride) was studied. Since extraction of oestrogens from urine would involve their exposure to air, light, and normal room temperatures, no attempt was made to protect the oestrogen solutions from such influences.

Stock solutions of stilboestrol, dienoestrol, and *iso*dienoestrol were prepared and allowed to stand in colourless glass flasks under ordinary laboratory conditions. Exposure to direct sunlight was avoided. Samples were withdrawn, initially and at intervals, and their chromogenic power towards the antimony reagent determined. The initial value, determined as soon as possible after solution had been achieved, was taken as indicating the full chromogenic power of the oestrogen initially present. Later values were recorded as percentages of this initial reading. The curves shown in Fig. 4 were constructed from mean values determined in a number of separate experiments. It is clear that dienoestrol and *iso*dienoestrol in solution rapidly lose their chromogenic power, while that of stilboestrol is maintained at its initial level for a comparatively long period. The stability of dienoestrol was tested in a number of solvents, and in every case the chromogenic power towards the antimony reagent decreased with increasing age of the solution.

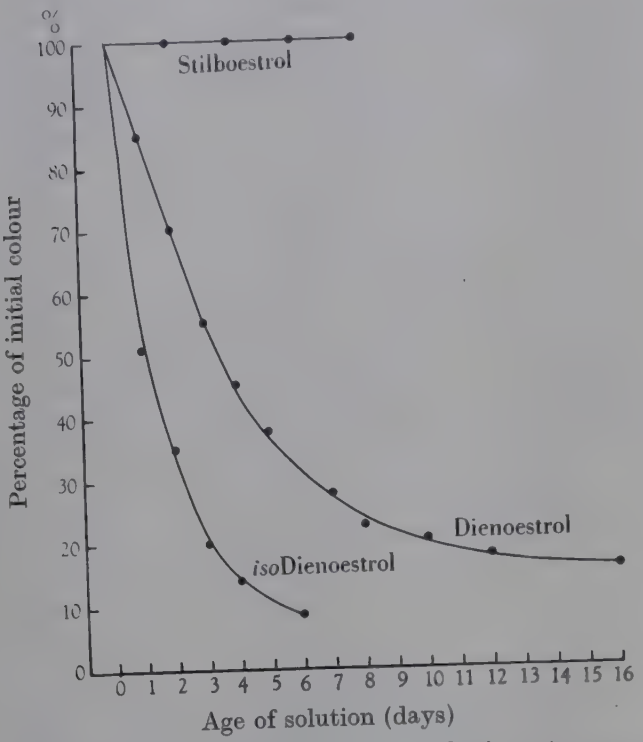

Fig. 4. Effect of age of solution of synthetic oestrogens on the reaction with $SbCl_5$. Chromogenic power expressed as percentage of initial colour value.

Application of Malpress's nitro reaction to the determination of synthetic oestrogens in chloroform and in ethylene dichloride solution

Simultaneously with the above experiments, the chromogenic power of the ageing stock solutions of stilboestrol, dienoestrol, and *iso*dienoestrol was measured by Malpress's method. Samples were withdrawn at intervals, the solvent evaporated at low temperature in a stream of nitrogen and the residue dissolved in glacial acetic acid. The subsequent procedure was as described by Malpress. The mean results of these experiments are shown in Fig. 5. The decrease in colour developed with increasing age of the solutions in the case of dienoestrol and of *iso*dienoestrol is again clearly shown, although the actual decrease at any given age is not so great as with the antimony reagent. The lowest value for dienoestrol (at 16 days) was 53·5% of the initial value, whereas the antimony reagent showed only about 15% of dienoestrol to exist unchanged at this time.

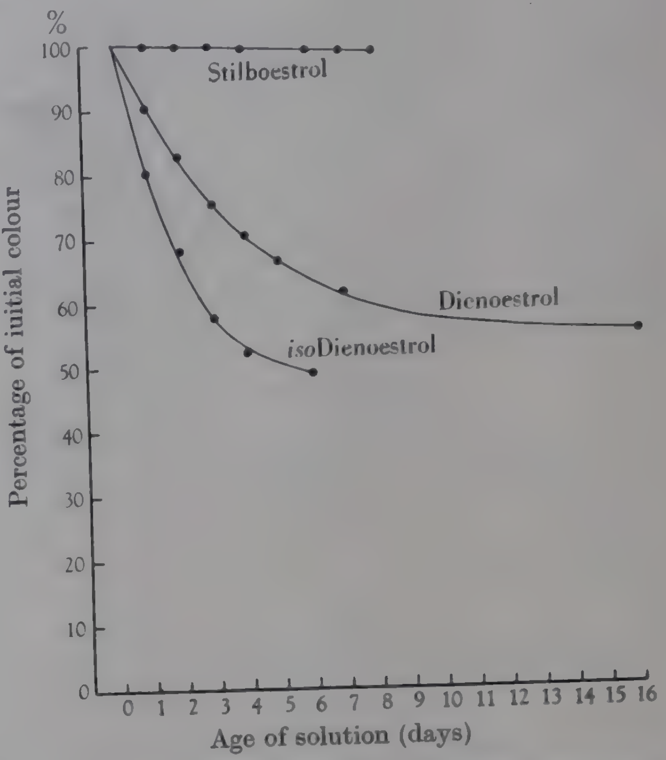

Fig. 5. Effect of age of solution of synthetic oestrogens on the Malpress nitro reaction. Chromogenic power expressed as percentage of initial colour value.

Mechanism of the 'decay' of dienoestrol solutions

It appeared probable that the explanation of the gradual loss of reactivity of dienoestrol towards antimony pentachloride might be a slow oxidation of the substance leading to disappearance of ethylenic double bonds. The integrity of these bonds is apparently essential for chromogenic power.

In order to test this possibility, solutions of dienoestrol in ethylene dichloride were prepared, and stored in the light and at room temperature but in the absence of air, achieved by thorough evacuation of the tubes containing the solution and subsequent sealing when all air had been displaced by solvent vapour. The same number of control (unsealed) tubes was stored under the same conditions. One sealed tube and one control tube were tested with antimony reagent at weekly intervals.

At the end of the experiment (21 days) no loss of chromogenic power had occurred in the dienoestrol in the sealed tubes, whereas the unsealed controls had shown the usual rapid 'decay'. It can be concluded, therefore, that the decay is caused by atmospheric oxidation of the oestrogen.

Application of the antimony reaction to the estimation of synthetic oestrogens in extracts of human urine

It was clear, from the experiments described above, that the problem of the detection and estimation of dienoestrol in human urine was likely to be a difficult one. The difficulties involved in the prevention of oxidation during the necessary hydrolytic and extraction processes appeared formidable. On the other hand, the proved stability of stilboestrol, and the high sensitivity of the reagent towards this oestrogen, suggested that it should be possible to obtain at least approximate measurements of the urinary excretion of stilboestrol in subjects undergoing treatment with this substance. Attention was therefore confined to urines likely to contain stilboestrol.

The complicating factor in the use of the antimony reagent on urine extracts is the presence of relatively large quantities of phenols. Small quantities of simple phenols (phenol or cresol) completely inhibit colour development. It was found, however, that the quantity of phenol present in the urine extracts could easily be reduced below the interference level by heating the extracts at 100° *in vacuo*. Recovery experiments with known amounts of stilboestrol (1–10 μg.), to which phenol or cresol had been added in amounts (5–10 mg.) comparable to those encountered in urine extracts, gave excellent results. The method finally adopted for investigating the stilboestrol content of urine was as follows:

(*a*) *Unhydrolyzed urine.* Fresh urine (100 ml.) was extracted with chloroform, the chloroform washed with water, dried, and distilled off *in vacuo*. The residue was treated as described below for the extract from hydrolyzed urine.

(*b*) *Hydrolyzed urine.* Urine (100 ml.) was acidified with 15 ml. of conc. HCl, 50 ml. of benzene added and the mixture refluxed for 3 hr. on the water bath. The benzene layer was then removed and replaced by a fresh 50 ml., and the mixture again refluxed for 3 hr. The combined benzene extracts were washed twice with water, the benzene separated, filtered and evaporated.

The residue was dissolved in 10 ml. of ethylene dichloride. 1 ml. of this solution was removed and diluted to 10 ml. with ethylene dichloride. This 10 ml. of solution served as a 'blank' for the colorimetric estimation; the urine extracts frequently had a red colour which contributed to the final red colour developed by the oestrogen, and it was necessary to correct for this.

A further 1 ml. of the original solution was transferred to a pyrex Thunberg tube which was then immersed in a boiling water bath and the solvent carefully evaporated at ordinary pressure. A vacuum was then applied (water pump) and the tube maintained in the bath for 15 min. The top part of the tube was carefully heated with a free flame to prevent condensation of the phenolic distillate.

After cooling the tube, the contents were dissolved in 1 ml. of ethylene dichloride and treated with 9 ml. of antimony reagent. The colour was allowed to develop at 30° for 15 min. and then measured (against the blank solution described above).

Urines from ten different patients were examined by this method. Eight of these subjects were receiving oral therapeutic doses of 5–15 mg. of stilboestrol daily. Two subjects had been implanted subcutaneously with 100 mg. of stilboestrol. In none of the extracts (unhydrolyzed or hydrolyzed) was unequivocal evidence of the presence of stilboestrol obtained. The colour developed was in all cases anomalous (i.e. brownish instead of red). On the other hand, small quantities of stilboestrol added to urine (either in solution in organic solvents or in aqueous solution as the sodium derivative) before hydrolysis and extraction could be recovered with approximately 100% accuracy. It is possible that the brown colour developed in these extracts may be due to decomposition products of stilboestrol present in the urines, since it was not observed in control experiments with normal urines.

DISCUSSION

Stilboestrol and dienoestrol are both widely used as therapeutic oestrogenic agents. In clinical practice dienoestrol is often used in preference to stilboestrol. This preference appears to be based on the belief that dienoestrol causes fewer side effects (nausea and vomiting) than does stilboestrol. Although this may be so when the two oestrogens are administered orally in the same amounts it is by no means certain that under these conditions the same oestrogenic effect is obtained.

The most important result of the present work is the demonstration of the great difference in stability between stilboestrol and dienoestrol. While the former substance has considerable stability in solution and resists oxidation, solutions of dienoestrol undergo very rapid atmospheric oxidation. This oxidation leads to loss of chromogenic potency towards both the Dingemanse and the Malpress reagents, and at the same time there is a corresponding decrease in oestrogenic potency. Since this oxidative destruction occurs readily in a variety of solvents (including aqueous solvents) it would clearly be difficult to prepare solutions of dienoestrol of known oestrogenic potency for therapeutic purposes unless some antioxidant were incorporated in such solutions.

The attempts to demonstrate the presence of stilboestrol in the urine of subjects undergoing treatment with relatively large amounts of this oestrogen, which have been described above, must be regarded as preliminary experiments. However, if account is taken of the high sensitivity of the method used, the total amount of stilboestrol (conjugated or free) in these urines must have been very small. The urine extracts gave anomalous colours (brown). But if the total absorption of green light by such solutions is taken as representing the maximum amount of stilboestrol which could be present (an obvious overestimate), the total daily urinary excretion of stilboestrol in patients receiving a daily oral dose of

15 mg. did not exceed 5% of the dose given. Since this figure is, admittedly, an overestimate, the results indicate that, broadly speaking, none of the administered stilboestrol was present in the extracts. This does not, of course, imply that the urine did not contain oestrogenic substances. It is possible that metabolites of stilboestrol were present, still possessing oestrogenic activity, but whose structure precluded the formation of red-coloured products with antimony pentachloride. The formation of the anomalous brown pigments by such urine extracts may be significant in this connexion.

The negligible amounts of free stilboestrol found in the extracts of unhydrolyzed urine in the present experiments are possibly to be expected by analogy with other compounds and with the results of experiments with stilboestrol in other species. The main excretory product might well be a conjugated product. Some observations of Malpress are of interest in this connexion. Malpress (private communication) has so far found it impossible to estimate the ether-insoluble forms of stilboestrol in cow's urine, owing to the complete destruction of this oestrogen when hydrolysis by hydrochloric acid takes place *in the presence of other urinary constituents*. On the other hand, as he has shown (Malpress, 1946), hydrolysis of the glucuronide by hydrochloric acid *in simple solution* gives recoveries of the oestrogen which closely approximate those which can be calculated from postulates as to the action of hydrochloric acid on pure stilboestrol. The failure to demonstrate conjugated stilboestrol in the present experiments may be due to similar causes.

SUMMARY

1. The behaviour of stilboestrol, dienoestrol, and *iso*dienoestrol in Dingemanse's antimony pentachloride reaction and Malpress's nitro reaction has been examined.

2. In solution in a variety of solvents, stilboestrol is stable, but dienoestrol and *iso*dienoestrol are unstable. This instability of dienoestrol and of *iso*dienoestrol in solution arises from their susceptibility to rapid atmospheric oxidation.

3. Attempts to demonstrate the presence of free or conjugated stilboestrol in the urine of subjects undergoing treatment with large therapeutic doses of this oestrogen failed to reveal the presence of significant amounts.

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The Osmotic Pressure of Insulin Solutions

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Sjögren & Svedberg (1931) examined solutions of crystalline insulin in the ultracentrifuge. From determinations of the sedimentation constant they concluded that it is a homogeneous protein, stable between pH 5 and 7. From sedimentation equilibrium measurements they calculated a molecular weight of 35,000. Polson (1937) determined the diffusion constant of insulin, and calculated from this ($D_{20} = 8{\cdot}2 \times 10^{-7}$) and Sjögren & Svedberg's sedimentation constant ($S_{20} = 3{\cdot}47 \times 10^{-13}$) the molecular weight 40,900. From Crowfoot's (1938) crystallographic measurements the weight of the dry unit cell is about 36,000.* The unit cell has trigonal symmetry, and the molecular weight of insulin in the crystal is therefore 36,000 or 12,000.

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* The value quoted by Crowfoot (1938) for the dry unit cell weight is 37,600. This has been calculated on the assumption that the moisture contents of air-dried insulin crystals are 5·35%. Miller & du Vigneaud (1937) pointed out the difficulties of obtaining accurate values for the moisture contents of insulin crystals and found 10·2% as the result of repeated analyses. The present author analyzed five samples of air-dried insulin crystals and found variations between 10 and 12% for the moisture contents. Completely dried crystals, if left at room temperature, absorbed water until they contained from 10 to 11%.

The molecular weight of insulin was generally accepted to be about 36,000 up to the publication of the work of Miller & Andersson (1942), who carried out a thorough study of the sedimentation and diffusion constant and calculated the molecular weight 46,000 from their data ($S_{20} = 3{\cdot}55 \times 10^{-13}$ and $D_{20} = 7{\cdot}53 \times 10^{-7}$).

Gutfreund & Ogston (1946) re-examined the sedimentation constant with a view to observing any changes of insulin in alkaline solutions and on dilution. They found that $S_{20} = 3{\cdot}35 \times 10^{-13}$ over a range of pH from 6·8 to 9·6 and at protein concentrations from 0·1 to 1·0 %. From this sedimentation and the diffusion constant ($D_{20} = 7{\cdot}0 \times 10^{-7}$) obtained by Gutfreund (unpublished) the molecular weight 46,600 can be calculated.

On account of the discrepancies between the data for the molecular weight ranging from 35,000 to 46,000, a detailed study of the osmotic pressure of insulin solutions was undertaken. No previous osmotic pressure measurements have been recorded for this protein, probably because of the sparing solubility near the isoelectric point and the indication of dissociation outside the pH range 4·5–7·5 (Sjögren & Svedberg, 1931).

Osmotic pressure and membrane potentials

The results of osmotic pressure measurements were used for the calculation of the molecular weight of insulin. As shown by Adair & Robinson (1930), this can be done by the use of the following equation:

$$P - P_i = P_p = RTg_p\, M_p,$$

where P = observed osmotic pressure; P_i = ion pressure difference; P_p = pressure due to protein, i.e. colloid osmotic pressure; g_p = osmotic coefficient (correcting for repulsive and attractive forces); and M_p = mol. protein/l. of solvent. The correction factors can usually be eliminated simultaneously by use of the equation

$$M = 10RT/\pi_0,$$

where M = molecular weight; $\pi_0 = P/C$ extrapolated to zero concentration; C = concentration of protein in g./100 ml. of solution. (The factor 10 in the above equation brings this up to conc./l. of solution.)

If the charge of the protein particles in solution is high and the salt concentration of the solvent buffer low, P_i may still be significant at low-protein concentrations. This has to be checked by membrane potential measurements or chemical analysis of diffusible ion concentration on both sides of the membrane (Adair & Robinson, 1930; Adair & Adair, 1934). For the work described in this paper the membrane potential was used for the calculation of P_i after the equation developed by Adair (1928):

$$P_i = 15{\cdot}4\, E^2\, \Sigma C_i'' Z_i^2,$$

where P_i is the ion pressure difference in mm. of mercury; E is the membrane potential, and $\Sigma C_i'' Z_i^2$ is the sum of the buffer ion concentration in the dialysate, multiplied by the square of their valence. All the experiments described in this paper were carried out on solutions of protein concentration below 1 %, and under those conditions the factor g_p can be neglected for nearly spherical molecules, such as insulin. Throughout this paper we have therefore used the simple formula $P_p = P - P_i = RT\ (C/M)$ for molecular weight calculations.

EXPERIMENTAL

Two types of osmometer were used for the experiments described in this paper. The simple apparatus developed by Adair (1925) was employed for the first two series of measurements. The osmotic pressure is read directly from the height of the solution in the capillary. When equilibrium is reached the capillary tube is removed and two samples of protein are taken from the membrane. The first is used for capillarity measurements and the second for estimation of protein concentration. Wherever required, membrane potential measurements were then carried out on the remaining solution. The technique described by Adair & Adair (1934) was followed in every detail for the measurement of membrane potentials.

The corrections for capillarity in the experiments recorded in Tables 1 and 2 are between 13 and 20 mm., depending on the inner diameter of the capillary. A study of the duplicate measurements of capillarity suggested that it would not be possible to get accurate results from osmotic pressure experiments on solutions containing less than 0·5 % protein, as a small error in the correction would have a large effect on the molecular weight calculation. If very wide capillary tubes are used the correction can be reduced, but equilibration is prolonged considerably. A new osmometer, employing toluene as pressure head in the capillary, has been developed by Mr G. S. Adair (to be published). With this apparatus much more reliable capillarity corrections can be made. The osmometers have been used according to the technique developed by Adair (personal communication), and the results of measurements on dilute solutions are recorded in Tables 3 and 4.

The following insulin preparations were used: (1) crystalline insulin from Boots Pure Drug Co. Ltd., (2) Squibb crystalline insulin. Nitrogen, moisture and ash contents of all samples of insulin received were examined. For the nitrogen estimation the micro-Kjeldahl procedure, with all precautions suggested by Chibnall, Rees & Williams (1943), was used. This analysis was important as the concentration of insulin in solutions after osmotic pressure measurements were calculated either from nitrogen contents or from refractive index difference (Δn) measurements. Δn was estimated in a Zeiss interferometer which was standardized with insulin solutions, their concentration previously being determined from micro-Kjeldahl nitrogen. The values obtained for the nitrogen contents of three samples of Boots crystalline insulin and one sample of Squibb crystalline insulin were all within 1 % of the value quoted by Chibnall *et al.* (1943), i.e. 15·4 % nitrogen in moisture- and ash-free protein.

The samples of protein were the same as those used by Gutfreund & Ogston (1946) for their ultracentrifuge experiments and shown to be homogeneous. Examination in the Tiselius electrophoresis apparatus also confirmed their purity.

Table 1. *Molecular weight of Boots crystalline insulin in buffer* pH 7·0

(In 0·1 M-phosphate; temp. 18°.)

P_p corrected to 0° (mm. H_2O)	Protein conc. (%)	Mol. wt.
9	0·14	36,000
9	0·145	35,300
11	0·18	37,800
14	0·21	34,700
14	0·22	36,400
	Mean	36,060 ± 1080 (S.D.)

RESULTS

The major difficulty encountered during all the experiments on insulin solutions was the low solubility and with that the measurement of very small osmotic pressures. The first series of measurements was carried out on solutions of insulin in 0·1 M-phosphate buffer (pH 7·0) at 18°. Though it was possible to obtain supersaturated solutions, containing 1% protein, under these conditions precipitation occurred during osmotic equilibrium, and the final protein concentration was always below 0·25%. Table 1 shows the results of this first series of measurements, giving a mean molecular weight for insulin of 36,000. The membrane potential under those conditions of protein and buffer concentration was measured and found to be negligible

$$(P_i < 0·05 \text{ mm. } H_2O).$$

Table 2. *Molecular weight of crystalline insulin preparations in buffer* pH 7·1

(In 0·01 M-phosphate; temp. 0°.)

Preparation	P (mm. H_2O)	P_i (mm. H_2O)	P_p (mm. H_2O)	Protein conc. (%)	Mol. wt.
Boots	23	2	21	0·46	51,000
	42	9	33	0·64	45,000
	35	7	28	0·58	48,000
	43	10	33	0·70	49,000
	36	7	29	0·58	46,000
	47	12	35	0·77	51,000
	40	8	32	0·60	43,000
	34	7	27	0·58	50,000
Squibb	51	13	38	0·77	47,000
	35	3*	32	0·65	47,000
				Mean	47,700 ± 3000 (S.D.)

* This experiment was carried out in 0·04 M-phosphate buffer pH 7·1.

Table 3. *Molecular weight of Squibb crystalline insulin (temperature* 0·05°)

P_p (mm. H_2O)	Protein conc. (%)	Mol. wt.
A. In phosphate buffer (0·02 M, pH 7·0).		
6·4	0·112	40,800
9·2	0·154	38,800
8·8	0·164	42,800
7·7	0·126	38,200
6·5	0·116	41,200
8·4	0·146	40,300
7·7	0·132	38,800
	Mean	40,300 ± 1400 (S.D.)
B. In phosphate buffer (0·02 M, pH 7·5).		
7·6	0·137	41,700
7·1	0·115	37,800
10·0	0·180	41,500
	Mean	40,300 ± 1800 (S.D.)

Table 4. *Molecular weight of Squibb crystalline insulin*

(In 0·02 M-phosphate buffer pH 7·0; temp. 20·5°.)

P_p (mm. H_2O, corrected to 0°)	Protein conc. (%)	Mol. wt.
8·1	0·122	34,900
9·6	0·136	30,500
9·4	0·143	35,400
9·8	0·115	27,100
10·2	0·132	39,800
10·2	0·143	32,200
10·8	0·159	34,100
	Mean	32,000 ± 2800 (S.D.)

It was observed that, on reducing the phosphate concentration of the buffer from 0·1 to 0·01 M, solutions containing up to 0·8% protein could be obtained at pH 7·0 and a temperature of 0°. Table 2 shows the results of a series of experiments carried out under those conditions. As can be seen the membrane potential effect, and with that the excess pressure of diffusible ions, becomes significant, both on account of higher protein concentration and of the reduced salt concentration in the buffer. The standard deviation from the mean molecular weight, 47,700 ± 3000, is much larger than that obtained from measurements on ovalbumin and lactoglobulin (Gutfreund, 1944, 1945), but this is due to the relatively small pressures measured and also due to the correction for P_i which, changing with the square of the membrane potential, may account for much of the deviations.

It was thought possible that the difference between the molecular weight calculated from measurements on solutions between 0·5 and 0·8 % protein and on solutions containing 0·15–0·25 %, may be due to dissociation of insulin on dilution. The accuracy of the results of Table 1 can be disputed on account of the difficulties of capillarity measurements in the type of osmometer used. The toluene osmometer, mentioned above, was therefore employed for a series of experiments which is recorded in Table 3. These results indicate, first, a dissociation of insulin on dilution, and secondly, that no further dissociation takes place on changing the pH from 7·0 to 7·5. As a further test for dissociation of insulin in dilute solutions, a series of measurements was carried out under identical conditions of pH and buffer and protein concentration, at a higher temperature. Table 4 shows the record of a number of experiments carried out at 20·5°. A marked increase of dissociation on raising the temperature can be observed.

DISCUSSION

In solutions containing 0·5–1·0 % protein the molecular weight of insulin is ~48,000. This is shown both by the results of osmotic pressure measurements and combined diffusion and sedimentation constants. In dilute solutions, containing less than 0·25 % protein, the molecular weight is lower, indicating dissociation. A further proof of the dissociation phenomena is given by the decrease of mean molecular weight with temperature. It has not been possible to upset the equilibrium completely to the side of the dissociation product and so to determine the size of the smaller unit, but both crystallographic evidence (Crowfoot, 1938) and chemical evidence (Chibnall, 1946) would be consistent with the assumption that subunits of molecular weight 12,000 are loosely bound to form insulin molecules. Further investigations on the molecular weight of the dissociation product will be described in a subsequent paper.

On the assumption that the dissociation of insulin on dilution is a reversible reaction $A_4 \rightleftharpoons 4A_1$ (A_4 is a molecule of weight 48,000 and A_1 a molecule of weight 12,000), the results recorded in Tables 3 and 4 are used for the calculation of the concentration of the A_4 and A_1 units, from the mean molecular weight at 0·5 and 20·5° respectively. Though particles of the type A_2 (molecular weight 24,000) and A_3 (molecular weight 36,000) may also be present, a correct measure of the number of bonds broken between these subunits is obtained by considering the equilibrium $A_4 \rightleftharpoons 4A_1$ only.

If x is the concentration of A_4 units and $(1-x)$ the concentration of A_1 units, the equilibrium constant $K=\frac{x}{(1-x)^4}$, x is calculated from the equation

$$\frac{1}{M}=\frac{x}{M_1}+\frac{(1-x)}{M_2},$$

where M = mean molecular weight, M_1 = molecular weight of A_4 unit, M_2 = molecular weight of A_1 unit. The free-energy change is calculated from

$$\Delta G=-RT\ln K:$$

at 0·5°: $x=0{\cdot}93$, $K=38{,}730$, $G=-57{,}292$ cal.

at 20·5°: $x=0{\cdot}83$, $K=994$, $G=-40{,}166$ cal.

Van't Hoff's reaction isochore can be used for the calculation of the total heat change ΔH, from the two equilibrium constants:

$$\ln\frac{K_1}{K_2}=-\frac{\Delta H}{R}\left(\frac{1}{T_1}-\frac{1}{T_2}\right),$$

$$\Delta H=-29{,}106 \text{ cal.}$$

From the relation $\Delta H=\Delta G+T\Delta S$ we can work out the entropy change ΔS. Taking a round mean value for $\Delta G=-48{,}000$ cal. (at 283° absolute),

$$\Delta S=+67 \text{ cal./degree.}$$

This is a first attempt to study reversible dissociation of proteins in relation to the energies involved. Though it is not justifiable to lay too much stress on the values obtained for ΔG and ΔH, they do give some indication of the type of reaction involved. This method of approach to the study of dissociation reactions is being continued and expanded for insulin and other proteins showing similar effects.

It is very likely that the dissociation effect, demonstrated here, accounts for the variation in the figures for the molecular weight of insulin, previously reported. Any measurements carried out in solutions would vary with concentration. The most interesting effect is the apparent formation of molecules consisting of three units (of molecular weight 12,000) in the crystal, while in solution the molecule can consist of up to four units. It is, of course, possible that still larger aggregates may form at higher concentrations. The calculation of molecular weight from crystallographic data is, under favourable conditions, the most accurate available, and it is a genuine difference in size of the molecule and not a disagreement of results, which is suggested.

Gutfreund & Ogston (1946) could not find any signs of dissociation in their ultracentrifuge experiments at concentrations below 0·2 %, but it is obvious that such small amounts as 10 or even 20 % of dissociation product would not be visible in the sedimenting boundary of such dilute solutions.

SUMMARY

1. It is shown that the molecular weight of insulin, calculated from osmotic pressure measurements on solutions containing 0·5–0·8 % protein at pH 7·0, is 48,000 ± 3000.

2. Insulin tends to dissociate on dilution to below 0·25 % protein concentration. The dissociation effect increases with temperature.

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3. Some preliminary calculations, on the energies involved in the dissociation reaction, have been carried out.

4. A possible cause for the discrepancies between previously reported molecular weight values is discussed.

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FREDERICK GOWLAND HOPKINS

Obituary Notice

FREDERICK GOWLAND HOPKINS, 1861–1947

PART 1. LIFE

Early life

Frederick Gowland Hopkins was born in Eastbourne on 20 June 1861. His father was not a professional scientist but had definite scientific leanings, being at one time associated with his second cousin, Sir Frederick Abel, in the latter's work on tides and currents. Unfortunately his father died while he was an infant and the rearing and educational guidance of the child devolved on the widow, who was assisted and guided by her brother, James Gowland.

From the age of six to ten Frederick Hopkins attended a dame school in Eastbourne; being the only day boy, he led a rather lonely life, his greatest recorded pleasure being in the use of his father's microscope about which he wrote:

> I felt in my bones that the powers of the microscope thus revealed to me were something very *important*—the most important thing I had yet come up against; so much more significant than anything I was being taught at school.

In 1871 the mother and son moved to Enfield to live with the uncle aforementioned and Hopkins's maternal grandmother. The boy was then sent to the City of London School, situated at that time in its old dismal surroundings in Honey Lane. This school seems to have done little to cater for the tastes and intelligence of the boy and he left at the age of fourteen, after several weeks of truancy, innocently occupied in country walks and in visits to museums and public libraries. This escapade, which he attributed to boredom, led to his removal to a private school where the educational advantages were of a low order. He left school for good at the age of seventeen, and, under pressure from his uncle, entered a firm of insurance agents. This unfortunate choice was not dictated by straitened means, but appears to have been made by a well-meaning but unimaginative relative as likely to provide a safe and satisfactory career. Hopkins stayed in this prison for six months only—long enough to learn the art of adding the three £ s. d. columns simultaneously—and escaped owing to the intervention of a family friend, who discerned in the boy an aptitude for science and advised his elders accordingly. Even now Hopkins was not sent to a university, though his father's friend and cousin, Sir Frederick Abel, whose advice was asked, said 'Cambridge'. This was, however, considered likely to provide too theoretical a training and Hopkins became an articled pupil in a private analytical laboratory run for profit. Here he spent the next three years in analytical practice, and records that, apart from manipulative skill, he learnt nothing worth while.

At the age of twenty one Hopkins became possessed of a small legacy from his paternal grandfather, and decided on his own initiative to embark on a scientific training at the Royal School of Mines in South Kensington. He was soon invited to become an assistant in the private laboratory of Dr Percy Faraday Frankland, where analytical work on railway material was in progress; however, at the end of six months this concern closed down and Hopkins was again at a loose end. This time he decided to work for the Fellowship of the Institute of Chemistry, and attended classes at University College, London, for that purpose. He records that he did exceptionally well in the examination and, as a result, was invited by Dr (afterwards Sir Thomas) Stevenson, the Home Office analyst and medical jurist, to become his assistant in his laboratory, then located in Guy's Hospital. Hopkins has stated that the five years he spent here were very happy ones; he learnt much, made lasting friends and worked on many famous and interesting cases. During his second year with Stevenson he and his colleague, Wade, decided to work for the London external degree; they both matriculated in 1887, entirely as the result of studies carried out in the evenings and on their daily railway journeys.

So far Hopkins's training had been purely chemical, but his position in Stevenson's department in Guy's Hospital brought him in contact with medicine and medical students. One of these (Sir) Charles Martin, recalls how he was wont to cultivate his taste for chemistry in the laboratory where Hopkins worked and to chat with the young analyst on problems of physiological chemistry which already fascinated both of them; it seems indeed to have been Martin himself who first suggested to Hopkins the idea of abandoning analytical chemistry and taking up research on physiological chemistry, and the urge to take this step evidently became stronger and stronger. In those days the main pathway into this field led through the study of medicine, and this consideration finally led Hopkins to interrupt his work as a chemist in order to undertake the arduous task of a medical training. In 1888, at the age of twenty seven he entered Guy's Hospital as a medical student, qualifying in science and medicine simultaneously. He passed the Intermediate B.Sc. in 1888 and B.Sc. in 1890 (honours in Chemistry and Physiology and gold medal in Chemistry). In the same year he passed the Intermediate M.B. and in 1894 the M.R.C.S. and L.R.C.P. and the London M.B.; his D.Sc. followed in 1900. During this period he held a studentship founded in memory of Sir William Gull.

Whilst still a student, and during the following years, he carried out research on a variety of subjects mainly arising from clinical studies and, after qualifying, soon established himself as a consulting clinician. He was also one of those who started and worked for the 'Clinical Research Association', an enterprise which undertook to examine and report on pathological, bacteriological and biochemical material sent in by medical practitioners—work now undertaken by public health laboratories but then uncatered for. Life at this time was very arduous but prospects were bright.

In 1898 Hopkins married Miss Jessie Stevens, daughter of Edward Stevens of St Lawrence, Kent. This event was of more importance to biochemistry than is often recognized. Had Hopkins added to the claims of a widowed mother (which he always paid in full measure) the exactions of a wife who was unable to appreciate his potential position as a scientist, or one who was apt to consider her own claims as important, the course of his career might well have been in clinical medicine, where he was already making both a name and an income, rather than in scientific research. Within a few months of their marriage, while they were living in Lincoln's Inn, the couple were faced with the parting ways. At the meeting of the Physiological Society held in Cambridge in 1898 Sir Michael Foster invited Hopkins to come to the Cambridge Physiological Laboratory, where he himself held the chair, to develop teaching and research in physiological chemistry. It was a difficult decision; all Hopkins's scientific ambitions and tastes urged him to accept the invitation, but common prudence insisted that he was already in a very favourable professional and financial position in clinical medicine, and leaving all this to go to Cambridge on a small income involved burning a veritable fleet of boats. Mrs Hopkins, herself connected with medicine through her profession as a nurse, might well have been expected to emphasize the latter consideration, but fortunately for science all her weight was on the other side, and it turned the scale; the Hopkins couple came to Cambridge in the same year. Throughout their lives Mrs (now Lady) Hopkins never wavered in supporting her husband in following, at whatever financial loss or risk, his career in research rather than anything else. Her own relations with his department were always smooth and easy; she was everyone's friend and nobody's enemy. Biochemists owe her more than they are ever likely to know.

The early Cambridge period

It need not be supposed that life in Cambridge was a change in all respects for the better. As usual in those days, his University stipend was quite inadequate for even the most modest requirements of a married couple. It was supplemented by Emmanuel College which appointed him supervisor of medical students and later (1906) Tutor in Science; this involved personal teaching both in physiology and anatomy; the latter was a real grind and in later years the Professor never alluded to it without making a wry face, and Lady Hopkins recalls how she was wont to sit up till 2 a.m. for fear her husband should neglect to make up the fire while he literally swotted up this uncongenial subject, which he had not touched since his student days.

These adverse circumstances remained unmitigated longer than might have been expected owing to the untimely death, in 1907, of Sir Michael Foster, who had been responsible for bringing Hopkins to Cambridge and was deeply interested in the success of the enterprise. But in spite of some attendant austerities things soon began to move along. The development of research during this period is peculiarly interesting but belongs to the later section of this article, but it must be recorded here that his University position was recognized and stabilized by the creation, in 1902, of a Readership in Physiological Chemistry, which at any rate implied that in his four years at Cambridge, Hopkins had convinced some authorities that the subject existed and was worthy of support. In 1905 he was elected to a Fellowship of the Royal Society. In 1910 his circumstances were eased by his election to a Fellowship in Trinity College and a Praelectorship in Physiological Chemistry. This enabled him to give up his arduous teaching in Emmanuel, which notified its appreciation of his value by electing him to an honorary Fellowship in the following year. Hopkins always recalled with gratitude his connexion with Emmanuel and the help it afforded him in his early years in Cambridge, and his elder daughter was married in the College Chapel. The connexion with Trinity, begun at this time, was also a great source of pleasure and satisfaction, a feeling which was surely fully reciprocated by those Fellows who enjoyed the wisdom of his counsels and the pleasure of his society. Hopkins speaks of his appointment to the Praelectorship thus:

> ...for me the election was salvation and my debt to Trinity is great indeed. For 26 years I have received all the great privileges that membership of the College confers and have been asked for no single service in return. I know well that my election was in the first place due to the

generous advocacy of Walter Fletcher. It is my hope that in any account of my career published after my departure the generosity of Trinity College will be emphasized.

Second Cambridge period. Recognition and growing success

In 1914 a special Chair of Biochemistry was created for Hopkins, but he did not at this time get a new building as was at first hoped; instead this went to Physiology whilst Hopkins, as representing the junior subject, had to content himself with the old building in Corn Exchange Street vacated by the senior science. Nevertheless he now had a chair, a professorial income and a department. He had established Biochemistry in Cambridge, indeed in England.

In the same year came the first world war, and Hopkins turned over his laboratory to the study of war problems, mainly nutritional. He became one of the original members of the Medical Research Committee (later Council) whose first Secretary was his friend and collaborator, Walter Fletcher; he worked on numerous committees, advising the Government on food policy and medical matters. By the end of the war Hopkins's scientific value was recognized far and wide.

In 1920, through the generosity of the Beit Trustees, he was able to expand his Department by the acquisition of the Balfour laboratory into which his rapidly increasing number of research workers was able to overflow.

Later Cambridge period. Achievement and honours

In 1921 part of the bequest of Sir William Dunn was devoted by the trustees to the building of a laboratory and the endowment of a Chair of Biochemistry in the University, and the subject was thus permanently established in Cambridge; the new laboratory was occupied in 1924. Honours and recognition now flowed thick and fast and it is impossible to enumerate them all. In 1921 he was invited to deliver the Herter lectures at Johns Hopkins University, and he and his wife made their first visit to the States, cementing earlier friendships and making many new ones. He was knighted in 1925, received the Copley medal of the Royal Society in 1926, and in the same year delivered the inaugural address to the International Physiological Congress in Stockholm. In 1929 he shared with Eijkman the Nobel Prize for Medicine. He was elected President of the Royal Society in 1931 and appointed to the Order of Merit in 1935.

In 1939 he underwent an operation after which his health woefully declined; he became increasingly feeble in body (though never in mind) and nearly blind. He carried on laboratory work, with the help of his assistant, James Morgan, till the end and his last seven papers were the result of work done in these difficult circumstances.

He died on 16 May 1947. His wife, Lady Hopkins, who contributed so much to all aspects of his life and cared for him with such skill and devotion during his last years, survives him. He leaves a son in the medical profession and two daughters, Barbara Holmes and Jacquetta Hawkes, distinguished in biochemistry and archaeology respectively.

PART 2. SCIENTIFIC WORK

It is convenient to divide Hopkins's scientific work into the same chronological periods as were used in the outline of his life.

Early life

As a boy Frederick Hopkins was interested in butterflies and beetles and his first published paper, written at the age of seventeen, was a letter to the *Entomologist* on the habits of *Brachinus crepitans* (Hopkins, 1878). His early entomological studies must have influenced his thoughts considerably for, apart from this letter, three out of his first six published papers were studies on the pigments of the *Pieridae*. This work culminated in a paper published in the *Philosophical Transactions of the Royal Society* (Hopkins, 1896) in which the white pigment of the common cabbage butterfly was identified with uric acid, and the fact that an excretory product finally turned up as an ornamental pigment emphasized. Actually the identification of the main pigment with uric acid was incorrect, a mistake which he recognized when he returned to the subject very late in his life (Hopkins, 1942), but its close chemical relation with uric acid and its appearance as an excretory product together with lepidoporphyrin, the yellow pigment of the related brimstone butterfly, in the rectum of the pupa shortly before the emergence of the butterfly struck him very forcibly. The work as a whole unites the skill of the analyst with the knowledge and outlook of the biologist, and already denotes the dual nature of the writer's scientific character.

Problems in uric acid excretion occupied a good deal of attention during this period, probably owing to the influence of Garrod. A reliable method was perfected for estimating uric acid in urine (Hopkins, 1893) and used in a study of uric acid metabolism (Hopkins & Hope, 1898–9). It was here shown for the first time that the nucleins of the diet were not alone responsible for the peak rise in uric acid and excretion after food.

A fine study on urobilin was done in conjunction with Garrod in this period (Garrod & Hopkins, 1896) in which Hopkins obviously contributed the method of separating urobilin in a purified state from normal and pathological urines, faeces and bile, and both contributors showed by the use of the spectroscope the identity of the products from the four sources; this paper is characteristic of Hopkins's exact description of the methods employed in the separation, giving at each stage the reasons for the precautions used and taking obvious pleasure in the technical details.

Hopkins began his protein work at this time with studies of the compounds formed by these substances with halogens (Hopkins & Brooks, 1897–8; Hopkins & Pinkus, 1898–9) and in preliminary accounts of the crystallization of egg albumin (Hopkins, 1898*a*). A comprehensive article on urine written for Schäfer's text-book also belongs to this period (Hopkins, 1898*b*).

Early Cambridge period

The first paper to be published after moving to Cambridge was the still famous description of the crystallization of egg albumin, the preliminary studies for which had been begun in London. Hopkins's interest in proteins had many facets; first, as the principal constituent of 'protoplasm' they demanded attention from anyone claiming to attack the chemistry of the living organism; secondly, so long as the chemistry of proteins remained vague, support was lent to those who claimed that the study of the chemistry of life was a hopeless endeavour. Hopkins doubtless felt that to obtain proteins in a crystalline form was a step towards reconciling chemists to undertake their study, though he himself entertained no illusions as to the significance of the crystalline state with respect to these substances. Having, however, replaced the crude procedure of Hofmeister by carrying out the precipitation of the protein by ammonium sulphate at a controlled pH using dilute acetic acid, he transformed a difficult and uncertain operation into one of ease and certainty, capable of giving high yields; this same method is in frequent use to-day in the purification of enzyme proteins. By carrying out measurements on the optical rotation and estimations of C, H, N and S on samples which had been subjected to a varying number of recrystallizations, Hopkins showed that he was dealing with a chemical entity; at that period this was a great advance in the study of proteins, and helped to eradicate the view that they were unstable entities of fluctuating composition. Having achieved this, Hopkins did not repeat the performance with other proteins, nor did he set his pupils to gain easy credit by crystallizing a series of these compounds as many a lesser man would have been tempted to do; instead he turned to other problems.

In the next three papers, also concerned with proteins, Hopkins had the collaboration of S. W. Cole who was then beginning his career in research and teaching. These form a logical series of great importance and interest, the stimulus for which arose from an amusing accident. It was customary in those days (and still is) for elementary students to be introduced to the study of proteins by performing a series of colour reactions. On one occasion the entire class failed to perform the Adamkiewicz reaction—then completely empirical—consisting in the production of a violet colour on the addition of dilute acetic followed by strong sulphuric acid. This led to the discovery that if the acetic acid was pure the test was always negative, but that the active agent could be separated from samples giving the test either by distillation or by crystallizing out the acetic acid. After an amusing and highly informative piece of research, the active agent was proved beyond doubt, by a number of rigorous tests, to be glyoxylic acid and the test thereafter became known as the glyoxylic test (Hopkins & Cole, 1901*a*). Having succeeded in identifying the active participant in the reagent, the same authors were moved to search for the group or unit in the protein molecule with which it reacted. This led to the famous isolation of tryptophan (Hopkins & Cole, 1901*b*). This work is too well known to need comment, but it may be noted that here Hopkins used for the first time his favourite precipitating agent, viz. 10% mercuric sulphate in 5% sulphuric acid.

The same authors (1903) sought to establish the identity of the new amino-acid by a highly original and biological method, viz. the identification of various products of its bacterial decomposition. Using as a substrate 2 g. tryptophan in 2% gelatin they obtained by the action of *Esch. coli*, aerobically, both indole and indolylacetic acid and by *Cl. chauvei* and *Esch. coli* anaerobically indolylpropionic acid. Unfortunately the three last compounds were, on the basis of early work, erroneously identified as their skatole isomers, i.e. skatolyl-2-carboxylic instead of β-indolylacetic and skatolyl-2-acetic in place of β-indolyl-propionic acids; this led to the formula first assigned to tryptophan being skatolyl-2-amino-acetic acid, a mistake which was only cleared up when tryptophan was synthesized from β-indolyl-aldehyde and hippuric acid (Ellinger & Flamand, 1907).

Hopkins's biological interests led him immediately to investigate the importance of tryptophan in mammalian diet (Willcock & Hopkins, 1907). Zein was used as the protein, this being known to be deficient in both tryptophan and lysine; young (and later adult) mice were the experimental animals. The results were without ambiguity:

> Zein, it is clear, has no power whatever of maintaining growth in the young animal, loss of weight begins the moment it forms the sole nitrogenous supply. The addition of the missing tryptophan group to the zein has, it is also clear, no power to convert such loss into equilibrium or

gain; a fact possibly due to other deficiencies in the zein molecule, such as the absence of lysine, or the lack of some other amino-acid not yet observed.

On the other hand the benefit of added tryptophan was clear; it almost doubled the survival period and eliminated the marked symptoms of lethargy noted on the unsupplemented zein. This study is important as introducing the knowledge that certain amino-acids are essential to the mammalian organism and, when absent from the protein food, can be supplied as such in the dietary.

Soon after this important work came the joint study with Fletcher on lactic acid in muscle (Fletcher & Hopkins, 1907). Fletcher had been working on this subject since 1898 and had already begun the study of CO_2 production in muscle on rational lines; when Hopkins joined in the work soon after coming to Cambridge a very fruitful collaboration began. The paper of 1907 is important for several reasons. By the use of ice-cold ethanol, lactic acid was extracted without stimulating the muscle and an important source of error avoided. Thus it was possible to demonstrate the accumulation of lactic acid in fatigue during anaerobiosis, and its removal in oxygen, and also the fact that the production of lactic acid in heat rigor is the same in resting muscle as in muscle which has been stimulated for 50 hr., whilst producing lactic acid over the whole of that period.

The great importance of this work is that it started the study of muscle fermentation and its relation to muscular contraction, a study which in the years that followed attracted many notable workers from all parts of the world, and developed into one of the most complete achievements of modern biochemistry providing, even to-day, material for work of major interest and importance. It also stimulated important work on biological oxidations.

Between 1906 and 1911 Hopkins published no major work, but there is evidence that he was employed in nutritional experiments which remained unpublished. In 1911 came a fine study on the Bence-Jones protein (Hopkins & Savory, 1911); this comprised a complete examination of the amino-acids of the protein by the methods of Kossel and of Fisher in addition to some originated by himself. Physical and analytical studies were also made, the whole illustrating Hopkins's wide command of technique. To all this were added important metabolic studies on the relation of the Bence-Jones protein in the nitrogen metabolism of the patient.

In 1912 was published what was perhaps the most famous of Hopkins's contributions to science, entitled 'Feeding experiments illustrating the importance of accessory factors in normal dietaries' (Hopkins, 1912). This work, as is invariably the case with communications of major importance, did not appear unheralded. The idea of deficiency diseases had been in the air for some time; Eijkman in 1890 and Grijns in 1901 had established the fact that beriberi was a deficiency disease due to a diet of over-milled rice; and in 1914 Funk was attempting the isolation of the active substance from rice polishings. Scurvy was believed to be due to a dietary deficiency owing to the work of Holst and Frölich. More historically startling is the fact that already in 1888 Lunin, working in Bunge's laboratory in Basle, had found that mice fed on the proteins, fats, carbohydrates and salts of milk failed to survive and concluded that milk must contain unknown substances necessary to life. Later Socin (1891), working in the same laboratory, showed that milk and eggs in small quantities rendered the synthetic diet adequate for growth and maintenance. Both these papers remained quite unnoticed, and in his Nobel Lecture of 11 December 1929 Hopkins discusses this misadventure at some length. Much later, in 1905, Pekelharing pointed out that there existed in milk a substance which, when present only in very small amounts, is of paramount importance in nutrition. Even at this late date Pekelharing's work attracted no attention, probably because it was written in Dutch and failed to get into the abstracts.

For many years Hopkins had had in mind the possibility that something more than pure proteins, fats, carbohydrates and salts might be necessary for growth and even for maintenance. He used to relate how, when a student at Guy's Hospital, two sailors were brought in with scurvy, and lay in adjacent beds while subject to the citric acid treatment used at that time. One day a relative of one of them brought him a bag of oranges; the subsequent spectacular recovery of the fortunate recipient did not escape the eye of the medical student, and seeds of doubt on accepted principles of diet were then sown to bear fruit later. Already in 1906 Hopkins in addressing the Society of Public Analysts committed himself to the following statement:

> No animal can live upon a mixture of pure protein, fat and carbohydrate, and even when the necessary inorganic material is carefully supplied the animal still cannot flourish.

In his Nobel Prize lecture (Hopkins, 1930) he admits to delaying the results of his earlier experiments owing to the

> great temptation to endeavour to isolate the active substance or substances before publication and I can claim that throughout the year 1909 I was engaged upon such attempts though without success.

Considering the complexity of the water soluble B group of vitamins this failure is not surprising. In his famous paper of 1912 Hopkins showed that a dietary consisting of casein, extracted exhaustively with ethanol, starch, sugar, lard and inorganic salts was unable to maintain growth or support life in young rats, but became perfectly adequate when fresh milk amounting to 1–3 % of the whole diet was added. The great feature of the work was the complete adequacy of the controls introduced in the selection of the animals, and the certainty that incomplete ingestion or absorption were playing no part. As a perfect experiment of its kind it stands as a model.

Second Cambridge period

In 1916 Hopkins returned to the study of the effects of individual amino-acids in the diet (Ackroyd & Hopkins, 1916), and made the important discovery that either arginine or histidine were necessary, the one being replaceable by the other; their role as precursors of allantoin was also shown. Throughout the period of the first world war, much of Hopkins's time and energy was occupied in popularizing the conception of the complexity of dietetic requirements, and the necessity for the supply of special molecular configurations, whether as vitamins or amino-acids, in opposition to the older notion that calories and proteins alone were the prime considerations in compiling an adequate diet.

Later Cambridge period

Towards the end of the first world war, Hopkins had become interested, through some experiments on the sulphur compounds of the cell, in a substance recognized as containing an SH group, found to have a very wide distribution in growing cells of both plants and animals. In 1921 he succeeded in isolating the substance from yeast and published a paper entitled 'An autoxidizable constituent of the cell' (Hopkins, 1921*a*). Hopkins called his substance glutathione, at that time believing it to be a dipeptide of glutamic acid and cysteine. This discovery inaugurated a long series of studies, both by Hopkins himself and by numerous pupils, on cellular oxidations in which this important peptide played the part of catalyst, and certainly orientated the activities of his department in the direction of biological oxidations for many years. In 1927 the constitution of glutathione was challenged by Hunter & Eagles, and Hopkins set about a reexamination of its structure (Hopkins, 1929). In the course of this work he completely changed the method of isolation, using a new and very beautiful technique which consisted in the precipitation of the peptide as a cuprous salt by the addition of cuprous oxide in the presence of $0{\cdot}5\text{N-}H_2SO_4$. The peptide came down in a high state of purity and proved to be a tripeptide of glutamic acid, glycine and cysteine. This discovery put the study of glutathione on a new and sounder footing; it also provided an example of a new and very elegant method for the isolation of the substance.

In 1921 Hopkins crystallized his views on biological oxidations, including the functions of glutathione, and on the position of muscle chemistry in the two Herter lectures which he delivered before the Johns Hopkins University in the spring of that year; on the same visit to the States he received the Chandler medal and devoted the address delivered on that occasion to the nutrition problem (Hopkins, 1921*b*, *c*; 1922). The lectures familiarized American biochemists with Hopkins's individual and original approach to the subject and resulted in a considerable flow of American workers to his laboratory in the succeeding years.

In 1923 he returned to his purine studies and, in collaboration with Morgan & Stewart, published the classical paper on the oxidation of xanthine and hypoxanthine to uric acid by the enzyme xanthine oxidase found in milk and animal tissues (Morgan, Stewart & Hopkins, 1922).

From 1931 to 1936 Hopkins was so much occupied with his duties as President of the Royal Society that his personal experimental work was sadly interrupted, and his published work during this period consisted largely of lectures and addresses. He returned to his laboratory in 1937, and then worked largely on problems connected with the function of thiol groups in enzymes, and drew attention to the part they play in maintaining active substances in the reduced condition in the cell.

In 1939 his period of bad health and failing eyesight set in, and he was hampered in his choice of subjects of research for this reason. In 1942 he returned to the study of butterfly pigments, and published an interesting paper on the pterins and their oxidation products (Hopkins, 1942). In his last years many of his earlier studies were resumed. Glutathione appears in two papers; in the first (Hopkins & Morgan, 1943) it was shown that, in many seeds, glutathione appears and increases in concentration in the early stages of germination. From 1·1 kg. of germinating pea seeds it was found possible to isolate sufficient glutathione in the crystalline state to serve for identification.

The second paper concerned the distribution of glyoxalase and its specific coenzyme, glutathione, in a large number of plants and invertebrate animals. Both the enzyme and coenzyme were found in almost every plant and animal tissue examined (Hopkins & Morgan, 1945). It is clear from the paper, and was emphasized in private conversation, that the discovery of the wide distribution of this enzyme throughout many divergent groups of organisms, whilst its natural substrate remained uncertain, was a source of worry and perplexity to Hopkins, and he earnestly recommended the problem to the notice of research workers. The fact that a function of glutathione is so closely involved in the problem doubtless increased its fascination for him.

In 1944 appeared a study of hypoxanthine, recalling Hopkins's interest in purine metabolism. This work was done in collaboration with Mrs Simon-Reuss who brought her experience of tissue culture to bear on the problem. Here it was shown that the addition of hypoxanthine in a concentration of 15 μg./ml. to the tissue culture media very markedly increased both the rate and duration of the tissue growth whilst adenine had no such effect, suggesting that the hypoxanthine supplement enabled the cells to synthesize nucleins more readily (Hopkins & Simon-Reuss, 1944). In 1945 Hopkins published work in which he examined the possibility that refection might have been in part responsible for his results in the vitamin work of 1912 (Hopkins & Leader, 1945). Here, using a technique due to Miss Leader, the phenomenon of refection was completely eliminated by the administration of filter paper as roughage. Rats so fed never showed refection owing, apparently, to the rapid removal of the undigested starch from the gut and the consequent prevention of excessive proliferation of bacteria and yeasts in the caecum. With animals so treated the effect of 3 ml. of milk/rat/day was as effective in maintaining growth as in the experiments of 1912, whilst the decline in the absence of the milk was equally unequivocal. In this study the reason for the association of refection with the use of potato starch as the main carbohydrate source was carefully studied, but no satisfactory solution to this problem was found.

A study on glyoxalase (Hopkins & Morgan, 1947) was in the press when Sir Frederick Hopkins died.

PART 3. TEACHING AND SCIENTIFIC INFLUENCE

A man's scientific achievement is the combined result of his scientific ability and his character. In no one is this more clearly exemplified than in Sir Frederick Hopkins. On looking back over the years it seems that he was destined from the first to occupy a unique position in science. He united an aptitude for chemistry, brought out by his training as an analyst, with an intense appreciation of biology; this is especially apparent in his early papers. To these qualifications he added a consciousness of having a mission with respect to the development of biochemistry, and a scientific insight amounting to genius. When he entered the field, biochemistry in England lagged far behind the developments on the continent. There, such workers as Kossel, Salkowski, Hoppe-Seyler and Emil Fischer had produced brilliant studies on compounds of importance in the animal and plant; no one of the calibre of these men had worked on similar problems in this country. But, conscious as Hopkins was of the high importance and value of these achievements in the structural chemistry of what may be termed the fundamental organic chemistry of the animal, it was not in this direction alone that he conceived biochemistry should advance. What fascinated him was not the static chemistry of the organic chemist, but the problem of the chemical changes which occur within the animal and plant cell and their relation to function. But the possibility of such studies appealed to very few persons capable of making them, and Hopkins set himself by achievement and by propaganda to induce a change of thought among chemists of his day. In an address to Public Analysts in 1906 he said:

> The scientific chemist—unlike his predecessors the pioneers of sixty or seventy years ago—has long since ceased to be much interested in the animal or the plant. Further, the triumph of synthetic work in advancing theory has led the pure chemist away from the special difficulties of analytical work. His extraordinarily developed technique concerns itself only secondarily and imperfectly with analytical studies of the kind still necessary in physiological problems, I mean the endeavours to identify and separate unknown substances with unknown properties present in complex mixtures. Such work requires special instincts and the pure chemist has largely lost them. He is but a poor analyst as the physiological explorer finds on turning to him for help (Hopkins, 1906).

Hopkins made also a bold plea for co-operation on equal footing of the chemist and the medical man:

> The care of the body in sickness must remain always an entire and carefully guarded prerogative of the physician; but the innate respect of the public...for the physician's calling has led to a somewhat illogical attitude and has tended to make sacrosanct not only the calling of the physician but the scientific material which he deals with.... The progress of scientific medicine demands a change here. While a large part of future scientific medical studies must always be carried out by men who, though medically qualified, have preferred the laboratory to practice...yet in a middle region these must be joined in their work by men whose primary qualifications are non-medical, men who, saved from the long years of clinical study, are able to bring well grounded laboratory knowledge and (I may add) a sufficient knowledge of the literature of pathology...to join their medically qualified confreres in attacking the huge problems which await solution (Hopkins, 1906).

Hopkins also felt the need to modify the attitude of physiologists towards biochemical studies; again his attitude is expressed best in his own words:

> I wish, however, to remind you of a mode of thought concerning the material basis of life which...was almost as inhibitory to productive chemical thought and study as any of the claims of vitalism. This was the conception of that material basis as a single entity as a definite though highly complex chemical compound. Up to the end of last century and even later, the term 'protoplasm' suggested such an entity to many minds.

To exemplify this he quotes Sir Michael Foster, whom he nevertheless revered, as saying,

> The oxygen which enters the muscle from the blood is not involved in immediate oxidations, but is built into the substance of the muscle. It disappears into some protoplasmic complex...we cannot yet trace the steps taken by the oxygen from the moment it slips from the blood into the muscle substance to the moment when it issues united with the carbon as carbonic acid. The whole mystery of life lies hidden in that process, and for the present we must be content with knowing the beginning and the end.

On which Hopkins comments:

> So long as the term 'protoplasm' retains a morphological significance as in classical cytology it may be even now convenient enough though always denoting an abstraction. Insofar, however, as the progress of metabolism...was ascribed in concrete thought to hypothetical qualities emergent from a protoplasmic complex in its integrity or when substances were held to suffer change only because in each living cell they are first built up, with loss of their own molecular structure and identity, into this complex, which is itself the inscrutable seat of cyclic change, then serious obscurantism was involved (Hopkins, 1935).

Hopkins disliked the conception of what he used to term the 'giant molecule' of living matter, within which mysterious chemical or pseudo-chemical events were supposed to occur, because he believed that it was inhibitory to biochemical thought and progress. So long as it remained, biochemistry, as he conceived it, would never develop because no one would attempt to solve the problems of chemical change within the body unless they were conceived of as ordinary chemical problems. Again and again Hopkins, in lectures, addresses and private conversations, returned to flog the 'giant molecule', just as Pasteur returned again and again to attack the heresy of 'spontaneous generation'. Until these ideas were removed from men's minds, Pasteur and Hopkins alike felt convinced that their respective sciences could not progress.

Hopkins early became convinced of the importance of chemical specificity in the organic molecules utilized by the animal. In a remarkable review paper in *Science Progress* 1906–7 he deals with the utilization of proteins by the animal, starting from the views of Liebig, to whom, as he humorously remarks, 'the fundamental aspects of protein metabolism in the animal appear to suggest no great difficulties' and 'whose answers to the questions partake of the lusty self confidence characteristic of biological science of his day'. He then proceeded to outline the evidence of recent work on the absorption from the gut, not of proteins, but of the constituent amino-acids, and outlined all the evidence extant at the time on protein digestion and utilization. In considering the 'remoulding' of the amino-acids into the proteins appropriate to the different organs of different animals he made the following prophetic utterance:

> Evidence is accumulating to show that there is a chemistry of species; that chemical differences in the protoplasm underlie morphological differences and that these differences concern in particular the complex and easily variable proteids.

The evidence accumulating with the years confirmed Hopkins in his conviction of the importance of specificity and by 1935 he was prepared to replace biogen or the giant molecule by a system in accord with modern research:

> Were it not equipped with catalysts, every living unit would be a static system.
>
> ...Catalysis is one of the most significant devices of nature, since it has endowed living systems with their fundamental character as transformers of energy, and all evidence suggests that it must have played an indispensable part in the living universe from the earliest stages of evolution.
>
> Life is manifested in chemical systems which it seems must have remained inert but for its influence. One would dearly like to know whether life at its origin was displayed by systems which could dispense with it, and if not what was the nature of the primitive catalyst.
>
> A molecule within the system of the cell may remain in an inactive state and enter into no reactions until at one... surface it comes in contact with an enzymic structure which displays certain adjustments to its own structure.
>
> An enzyme is in general adjusted to come into effective relations with one kind of molecule only or at most with molecules closely related in their structure....The one aspect of enzymic catalysis which for the sake of my theme I wish to emphasize is its high specificity (Hopkins, 1935).

Hopkins's own character contributed greatly to his success in persuading scientists to consider and ultimately to accept his views. He seldom antagonized opinion and never quarrelled; his relationship with his juniors was always stimulating and never authoritative. He could not tolerate strained relationship or personal quarrels and his department was singularly free from them. Nevertheless, he inhabited no ivory tower. Throughout his life he laboured unceasingly to establish biochemistry as a 'separate discipline' and, so far as Cambridge University was concerned, he succeeded. The early course of recognition of biochemistry as a subject is marked by the steps in the recognition of Hopkins in the University. As soon as he became master of an adequate laboratory (1924) he established Biochemistry as a subject for Part II of the Tripos and the formal teaching of advanced students began. In this course all senior members of the Department contributed their quota and young biochemists began to flow from Cambridge into scientific activities of various kinds. In 1938 Biochemistry was established as a subject for Part I of the tripos, giving to every science student in Cambridge the opportunity of acquiring an elementary knowledge of the subject should he so desire.

It need not be supposed that Hopkins achieved all this by lying on a bed of roses. He was personally beloved, but in establishing biochemistry he met with considerable opposition and criticism from the exponents of subjects upon the confines of which it bordered, and from non-scientists. But when the time came he never shrank from applying the necessary pressure and his many friends stood by ready to help. It is, moreover, noteworthy that at the time of his death only one other University in England, and one in Scotland, had established biochemistry as a subject for a degree, or attempted to share with Cambridge the task of training the biochemists of the future.

In 1925, the year Hopkins received his knighthood, there appeared in '*Brighter Biochemistry*', the illustrated journal of his laboratory, an excellent picture of our 'verray parfit gentil knight' on a war horse in a full suit of armour bearing a pennon inscribed with the motto 'Gluta thy Owne': the topical allusion is obvious, but the years have brought it a deeper meaning. In his early life Hopkins felt himself able to achieve a work for science in establishing biochemistry as a separate study and in stamping it with his own mark. He fulfilled this dream of his youth by thought, work and struggle, and showed a courage and tenacity of purpose somewhat at variance with his gentle, slightly hesitating, manner.

M. STEPHENSON

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The Selective Inhibition of Cholinesterases

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In recent years it has become apparent that cholinesterase preparations from different tissues exhibit fundamental differences in specificity and other properties (Alles & Hawes, 1940; Glick, 1941; Richter & Croft, 1942). Mendel & Rudney (1943) have suggested a classification of these enzymes into two main types: (1) the 'true' cholinesterases, active only towards certain esters of choline, and (2) the 'pseudo-cholinesterases', active towards both choline and non-choline esters.

In a further paper (Mendel, Mundell & Rudney, 1943) it is stated that the 'true' cholinesterases readily hydrolyze acetyl-β-methylcholine, but are inactive towards benzoylcholine, while the reverse is true for the 'pseudo' enzyme. This difference in substrate specificity is put forward as a practical criterion for the differentiation of the two types of enzyme. From their experiments with brain and blood of different species it appears that in general brain and red blood cells contain a preponderance of the 'true' while human serum or plasma, and that of several other species, contains chiefly the 'pseudo' enzyme. Another point of differentiation was revealed in the concentrations of acetylcholine at which maximum enzymic activity was obtained, the 'true' cholinesterase showing a maximum rate of hydrolytic activity at much lower concentrations of acetylcholine than those required by the 'pseudo' enzyme (Mendel & Rudney, 1943; Bissegger & Zeller, 1943).

Studies of the action of inhibitors on these enzymes have indicated further differences. Thus, Zeller (1942) found that the cholinesterase in human or rat brain is much less sensitive to inhibition by certain pyrazolone derivatives than the cholinesterase in human serum. Hottinger & Bloch (1943) examined the action of tri-*o*-cresyl phosphate on the cholinesterase activity of rabbit and human serum, liver and brain, but found that although the human tissues were in general more sensitive to inhibition by this compound than those of the rabbit, the three tissues of either species were inhibited to approximately the same extent. Zeller & Bissegger (1943), comparing the enzymes in human serum, brain and erythrocytes, showed that whereas morphine inhibited all three esterases to approximately the same extent, percaine inhibited the enzyme in serum more powerfully than that in brain or erythrocytes, while with caffeine the reverse was true; the concentrations of inhibitor used by these latter workers were, however, high. In a study of the action of certain prostigmine analogues Hawkins & Gunter (1946) found that the dimethylcarbamate of (2-hydroxy-5-phenylbenzyl)-trimethylammonium bromide (Hoffmann-La Roche, Nu 683) is, both *in vitro* and *in vivo*, a potent inhibitor of pseudocholinesterase, a concentration of 1×10^{-8}M producing 92 % inhibition of the hydrolysis of benzoylcholine by human plasma.

Di*iso*propyl fluorophosphonate, which, like other alkyl fluorophosphonates, possesses eserine-like properties in the animal body (McCombie, Adrian, Kilby & Kilby, 1941), was shown by Mackworth (1942) to inhibit serum cholinesterase at high dilutions. Mazur & Bodansky (1943, 1946) and Mendel & Hawkins (1947) have demonstrated that, while the esterase in human serum is highly sensitive to this compound, significantly higher concentrations are required to inhibit the cholinesterase in brain tissue.

It has been shown in this laboratory (Thompson, 1943, 1947; see also Gilman & Philips, 1946) that certain of the 'nitrogen mustards' (e.g. $\beta\beta'$-dichlorodiethyl-*N*-methylamine) are inhibitors of brain cholinesterase. Mackworth (1943), on the other hand, obtained only slight inhibition of the enzyme in serum. From these early results it seemed therefore as if $\beta\beta'$-dichlorodiethyl-*N*-methylamine might also be a differential inhibitor of the cholinesterases, but in the reverse sense to the fluorophosphonates, i.e. the 'true' enzyme in brain might be significantly more sensitive to inhibition by this compound than the 'pseudo' enzyme in serum. A more detailed comparison of the inhibition of certain cholinesterases by this compound has therefore been carried out with both acetylcholine and the 'specific' substrates of Mendel *et al.* (1943), and the results obtained with this inhibitor compared with those found with eserine and di*iso*propyl fluorophosphonate.

EXPERIMENTAL

Source of enzyme

Pigeon brain. Pigeons were killed by decapitation, and this was followed by immediate removal of the brain. The whole brain, wiped free from blood, was then dispersed by grinding in 10 times its weight of 0·024M-$NaHCO_3$, and filtered through muslin.

Human brain. Fresh samples of normal human cerebellum, removed during the course of operations for the excision of cerebellar tumours or chronic encapsulated abscesses, were kindly provided by Mr J. Pennybacker, F.R.C.S. After removal from the cranium the tissue was placed in ice-cold bicarbonate buffer. Large blood vessels and any adherent membranes were first dissected away, and the tissue washed free from blood clots with glass-distilled water. The weighed sample was then dispersed by grinding with bicarbonate buffer. Since appreciable quantities of blood were still present, the dispersion was strained through muslin, centrifuged, and the blood-stained supernatant liquid discarded. The precipitate was washed once more, and finally suspended in bicarbonate so that 2 ml. of the final suspension corresponded to 1 g. of original tissue.

Human plasma and serum. Freshly drawn blood was either added to potassium oxalate (1·0 mg./ml.) or allowed to clot and the clot broken up with a glass rod; separation by centrifuging followed in either case.

Human red blood cells. The cells obtained after removal of the oxalated plasma were washed five times with 0·9% NaCl, and finally lyzed by suspending in sufficient distilled water to make up to the original volume of blood.

All the enzyme preparations were stored, when necessary, at 0°, under which conditions their activity remained almost unchanged for several days. No attempt at purification was made. Plasma was used more often than serum in order to obtain more easily red cell preparations from the same blood sample. Massart & Dufait (1933*a*, *b*) have stated that oxalate is appreciably inhibitory towards horse-serum cholinesterase, due presumably to the removal of activating Ca^{++}; Gautrelet & Scheiner (1939), on the other hand, who used a biological method, have claimed that oxalate has no effect on serum cholinesterase. With human blood the final concentrations of oxalate used in this work produced less than 10% inhibition of the plasma enzyme. Further, the effects of the various inhibitors on serum or on plasma were the same, within the limits of experimental error. Consequently serum and plasma have been used interchangeably throughout the work.

Substrates

(1) *Acetylcholine chloride* (ACh) (British Drug Houses Ltd.).

(2) *Acetyl-β-methylcholine chloride* (MCh) (Savory and Moore Ltd.).

(3) *Benzoylcholine bromide* (BCh), prepared and kindly given to us by Dr A. H. Ford-Moore, Experimental Station, Porton.

Each substrate was dissolved in 0·024 M-$NaHCO_3$ immediately before use, and a sufficient volume of the solution added to the reaction mixture to bring the final concentrations to 0·006 M for ACh and BCh, and 0·03 M for MCh. Rough determinations of the Michaelis curves have shown that these concentrations lie upon the flat part of the curves obtained with the 'pseudo' enzyme, or are in excess of the peak concentrations for the 'true' enzyme (Mendel & Rudney, 1943).

Inhibitors

(1) *ββ′ - Dichlorodiethyl - N - methylamine hydrochloride* (DDM). Since this compound has been shown to undergo fairly rapid change in aqueous solution, with the formation of cyclic (ethyleneimonium) and hydroxy derivatives (Hartley, Powell & Rydon, 1942; Boyland, 1942; Cameron & Rydon, 1942; Bergmann & Fruton, 1944; Gilman & Philips, 1946), the solid was dissolved in 0·024 M-$NaHCO_3$ immediately before adding to the enzyme-bicarbonate mixture.

(2) *Diisopropyl fluorophosphonate* (DFP).

(3) *Eserine sulphate* (British Drug Houses Ltd.).

(4) *Trichlorotriethylamine hydrochloride.*

The latter inhibitors were also freshly made up in bicarbonate for each experiment.

Estimation of enzymic activity

Esterase activity was determined manometrically, using Ammon's (1933) adaptation of the Warburg technique, in which the acid produced by enzymic hydrolysis of the substrate is measured by CO_2 evolution from bicarbonate buffer at pH 7·4 (gas space filled with 95% N_2 and 5% CO_2). All measurements were made in duplicate at 38°. Non-enzymic hydrolysis was controlled by including a pair of vessels containing substrate but no enzyme.

The enzyme preparation together with the inhibitor was placed in the main compartment of the Warburg vessels, and the substrate in the side bulbs, the total volume being made up to 3 ml. by the addition of 0·024 M-$NaHCO_3$ to the main compartment. After gassing the vessels were placed in the thermostat, and 15 min. allowed for temperature equilibration; the substrate was then tipped in from the side bulbs and readings taken at 10 min. intervals. The inhibitors therefore remained in contact with the enzyme, and in the absence of substrate for 5–10 min. at room temperature, followed by a further 15 min. during the attainment of temperature equilibrium.

The first 10 min. readings have been neglected and the activity recorded over the period 10–40 min. Apart from errors introduced during the removal of the manometers from the bath in order to tip in the side-bulb contents, it was found that the non-enzymic hydrolysis was usually larger during the first few minutes. Further, in the case of reversible inhibition a measurable time must elapse to allow the new enzyme-substrate-inhibitor system to approach equilibrium (Eadie, 1942).

As far as was possible the quantity of enzyme taken was adjusted to that which gave, or would have given, 180–200 μl. CO_2/30 min. with acetylcholine as substrate.

RESULTS

To test the validity of the earlier work, the effects of different concentrations of DDM were first tested on the cholinesterase activity of pigeon brain and human plasma. These tissues were chosen, largely on account of their accessibility, as sources of the true and pseudo-enzymes respectively, and experiments have been carried out using both acetylcholine and the appropriate specific substrate of Mendel. In agreement with the results of Mendel *et al.* (1943) it was found that while both preparations readily hydrolyze acetylcholine, the brain enzyme is active against MCh but not against BCh, while the reverse is true for the serum enzyme. The inhibitions of enzymic activity produced are summarized in

Table 1. *Percentage inhibition by DDM of cholinesterase activity of pigeon brain and human serum*

(See p. 171 for explanation of abbreviations here and in following tables.)

		Concentration DDM ($\times 10^{-4}$ M)							
Enzyme source	Substrate	0·17	0·44	0·87	1·75	3·5	6·1	8·7	17·5
Pigeon brain	ACh	42	69	86	93	95	—	96	—
	MCh	39	62	84	93	—	—	—	—
Human plasma	ACh	—	—	8	16	31	47	56	62
	BCh	—	11	19	28	40	—	58	—

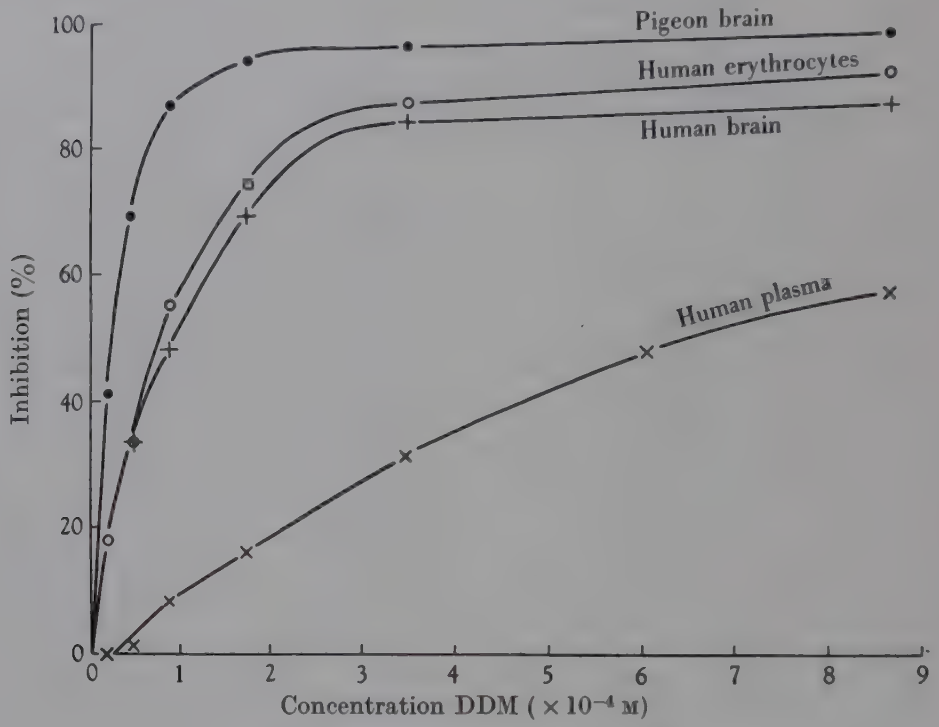

Fig. 1. Percentage inhibition of cholinesterases by DDM.

Table 1, the values for each concentration of inhibitor being the mean of several determinations, which in general agreed to within a few per cent.

The difference in the sensitivity to DDM of the cholinesterases in these two tissues is also shown in Fig. 1, from which it can be seen that, in order to produce 50 % inhibition of the enzymic hydrolysis of ACh, the concentration of inhibitor required for the plasma enzyme is about 30 times that required for pigeon brain. A similar difference exists when the specific substrates MCh and BCh are used with their respective enzymes, although appreciably more inhibition is obtained at low concentrations with the BCh-plasma enzyme system.

Since in these experiments, however, the two types of cholinesterase were derived from different animal species, the interpretation of the results is complicated by possible species differences in sensitivity to DDM. To overcome this, experiments were also carried out with preparations from human erythrocytes and human brain. The enzyme in each of these two tissues conforms to Mendel's criterion for a true cholinesterase in readily hydrolyzing ACh and MCh, while showing only a negligible activity with BCh. The results obtained with these tissues are shown in Table 2 (see also Fig. 1).

The human brain used in this work was in each experiment taken from the postero-lateral surface of the superior cerebellar lobule. Since the samples of tissue excised were not in each case identical

Table 2. *Percentage inhibition by DDM of cholinesterase in human erythrocytes and human brain*

		Concentration DDM ($\times 10^{-4}$ M)					
Enzyme source	Substrate	0·17	0·44	0·87	1·75	3·5	8·7
Human erythrocytes	ACh	18	34	55	74	86	90
	MCh	20	37	60	74	89	—
Human brain	ACh	—	34	48	69	83	85
	MCh	—	36	53	72	82	—

anatomically, considerable variations in activity were observed between them (one sample of cerebrum from the prefrontal cortex showed only negligible activity); weight for weight, the level of activity was about one eighth that of whole pigeon brain.

From Table 2 it will be seen that the activities of human erythrocytes and brain are inhibited to approximately the same extent by DDM; and although less sensitive to this substance than pigeon brain, each of these tissues is very considerably more sensitive than human plasma, 10^{-4}M-DDM causing 58 and 52 % inhibition with human erythrocyte and human brain respectively, but only 9 % with human plasma.

Differences in the response of an unpurified enzyme in different tissue preparations to the presence of a given concentration of an inhibitor might be due to variations in the amount of enzymically inert substances competing with the enzyme for the inhibitor. With this possibility in mind, and in view of the selective inhibition of the true and pseudo-cholinesterases by di*iso*propyl fluorophosphonate (Mazur & Bodansky, 1946; Mendel & Hawkins, 1947), a series of experiments was carried out under identical conditions using DFP instead of DDM, in order directly to compare the effects of these two inhibitors on our enzyme preparations. Table 3 summarizes the results obtained with DFP on the various tissues studied.

From these results, which confirm those of Mazur & Bodansky (1943, 1946) and Mendel & Hawkins (1947), it will be seen that with the same enzyme preparations functioning under the same conditions DFP inhibits selectively, but in the opposite direction to that of DDM, i.e. the plasma enzyme is very much more sensitive than that in brain to inhibition by DFP.

This contrasting behaviour of the cholinesterases from these sources is shown more clearly in Table 4, in which is given the concentrations (I_{50}) of these inhibitors which are required to produce 50 % inhibition of enzymic activity. The selective nature of the inhibitors may be conveniently presented as the ratio of these concentrations (I_{50} ratio).

In order to bring these results into line with the classical work on eserine we have also compared the effects of eserine sulphate on these enzymes under our conditions, using both acetylcholine and the specific substrates, and have confirmed the earlier findings (Mendel *et al.* 1943) that eserine shows no marked difference in its ability to inhibit the two types of cholinesterase; under our conditions 0·05 μg./3 ml. resulted in 75, 52 and 57 % inhibition of the hydrolysis of the specific substrates by the human plasma, pigeon brain and human red cell esterases respectively.

A few experiments have also been carried out with another of the 'nitrogen mustard' class of

Table 3. *Percentage inhibition of cholinesterases by DFP*

		Concentration DFP (× 10^{-8}M)									
Enzyme source	Substrate	3·6	7·1	9·1	14·4	18·2	29	36·4	59	73	144
Pigeon brain	ACh	—	11	—	19	—	30	—	52	—	85
	MCh	—	9	—	17	—	29	—	52	—	84
Human erythrocytes	ACh	8	24	—	45	—	77	—	97	—	—
	MCh	16	32	—	53	—	80	—	96	—	—
Human brain	ACh	—	—	35	—	61	—	80	—	96	—
	MCh	—	—	40	—	68	—	85	—	95	—

		Concentration DFP (× 10^{-8}M)						
		0·02	0·04	0·09	0·18	0·36	0·71	1·01
Human plasma	ACh	0	7	18	35	58	87	100
	BCh	—	—	16	34	79	86	—

Table 4. *Concentrations* (I_{50}) *of inhibitors required to produce* 50 % *inhibition of esterase activity*

Enzyme source	Substrate	I_{50} conc. of DDM (× 10^{-5}M)	I_{50} conc. of DFP (× 10^{-5}M)	I_{50} ratio (DDM/DFP)
Pigeon brain	ACh	2·4	0·057	42
	MCh	2·7	0·059	46
Human erythrocytes	ACh	7·4	0·015	493
	MCh	6·5	0·013	500
Human brain	ACh	8·7	0·013	669
	MCh	8·0	0·012	667
Human plasma	ACh	69	0·00029	$2·4 \times 10^5$
	BCh	58	0·00026	$2·2 \times 10^5$

compounds, trichlorotriethylamine hydrochloride. Although this compound is less selective than DDM in its effects on true and pseudo-cholinesterases, the enzyme in pigeon brain was again found to be more sensitive than that in human plasma.

DISCUSSION

The ability of certain compounds to inhibit the pseudo-cholinesterase at concentrations far below those required to affect the true cholinesterase has been well established. In addition to those substances already mentioned, Harris & Harris (1944) demonstrated the presence of another unidentified selective inhibitor of the pseudo-enzyme in certain curare preparations. A compound capable of inhibiting the true cholinesterase at low concentrations, while leaving the pseudo-enzyme relatively unaffected, does not, however, appear yet to have been described. Zeller & Bissegger (1943), it is true, showed that high concentrations of caffeine exerted this effect; their work, however, was carried out before the introduction of specific substrates for these enzymes, and, using a concentration of acetylcholine of 6·6 mM, they required a concentration of 6 mM-caffeine to produce 40 % inhibition of enzymic activity.

From the experiments described here, DDM, in so far as its effects on esterases are concerned, would appear to fulfil the role of a selective inhibitor of the true enzyme. It is, admittedly, a much less potent inhibitor than either DFP or eserine, but it must be remembered that its action has so far only been studied on unpurified tissue extracts. Further, few compounds of the 'nitrogen mustard' group have been even superficially investigated as cholinesterase inhibitors. It is not necessarily to be expected, therefore, that DDM is either the most potent or the most selective inhibitor among these substances. It is known that DDM also inhibits certain phosphokinases present in such tissue extracts (Dixon & Needham, 1946), so that it is not unlikely that the purified cholinesterase would show a significantly greater sensitivity to this substance. Even with our crude preparations, however, 50 % inhibition of the true cholinesterase has been obtained with concentrations varying from $2·5$ to $8·7 \times 10^{-5}$ M, whereas amounts of from roughly 10–30 times these concentrations are required to produce an equivalent inhibition of the pseudo-enzyme.

Of the large number of substances which have been described as inhibitors of cholinesterase it is now possible to distinguish three main types, examples of which are given below:

A. *Selective inhibitors for true cholinesterase:*

ββ'-Dichlorodiethyl-*N*-methylamine.
Caffeine.*

B. *Selective inhibitors for pseudo-cholinesterase:*

Alkyl fluorophosphonates.
Nu 683.
Curare (unauthenticated samples).
Percaine.*
Pyrazolone derivatives.*

C. *Inhibitors of both types of cholinesterase:*

Eserine.
Tri-*o*-cresyl phosphate.*
Morphine.*

* Not tested against specific substrates.

It is of interest that the values for the I_{50} concentrations of either DDM or DFP for the enzyme from a given source agree closely whether the substrate be ACh or the specific ester (Table 4), since, in the case of MCh, the concentration of substrate used differed widely from that of ACh.

The I_{50} concentrations of DDM and DFP given in Table 4 suggest strongly that, while the cholinesterase in human erythrocytes and human brain are at least very similar, they are fundamentally different from the enzyme in human plasma. Further, although the results obtained with pigeon brain differ appreciably from those obtained with the true cholinesterase in human tissues, they are in support of the general classification of the cholinesterases into true and pseudo types put forward by Mendel.

Inhibition by DFP is irreversible, and we have not found it to vary with changes in substrate concentration; DDM on the other hand appears to inhibit competitively, so that the I_{50} value will depend on substrate concentration. The I_{50} ratio for these two inhibitors will therefore also depend on substrate concentration as well as on the varying effect of enzymically inert substances present in the preparations which may be competing with the enzyme for each of the inhibitors. If, however, two more closely related inhibitors are used, interference by inert substances is less likely to affect them to different degrees. It is hoped, therefore, to extend this work to the study of two competitive inhibitors, in order to determine whether the I_{50} ratio can, under these conditions, be used as an aid in the characterization of related esterases.

SUMMARY

1. The inhibitory action of ββ'-dichlorodiethyl-*N*-methylamine on the true and pseudo-cholinesterases of brain, erythrocytes and plasma has been studied using both acetylcholine and Mendel's specific substrates, and the effects compared with those produced by di*iso*propyl fluorophosphonate and eserine.

2. ββ'-Dichlorodiethyl-*N*-methylamine has been shown to be a selective inhibitor of the true enzyme.

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The Formation of Nicotinamide from Nicotinic Acid by the Rat

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Part of the essential content of this paper has been communicated to the Biochemical Society (Ellinger, 1946*a*). The publication of details was postponed because the quantitative estimation of the nicotinamide formed, was carried out by an indirect biological method, which involved some uncontrollable factors. Confirmation of the results was desired using a more exact method which would permit the separate determination of nicotinamide and nicotinic acid in the same solution; but no suitable method has so far been found. No method based on the König (1904) principle is sensitive enough for the small amounts of nicotinamide and nicotinic acid to be measured in these experiments, and the errors involved in the estimation of nicotinamide by the differences found before and after alkaline hydrolysis are far too large. Scudi's (1946) method, based on the fluorimetric measurement of a pigment developed from nicotinamide with cyanogen bromide and alkali and extracted by *iso*butanol, permits the measurement of as little as 1 μg. of nicotinamide in the presence of nicotinic acid; the latter does not give a fluorescent product, but aneurin gives with the reagents a product of so high fluorescent efficiency that the method cannot be used for the determination of nicotinamide in the presence of aneurin. Microbiological methods using bacteria such as *Pasteurella* strains (Koser, Berkman & Dorfman, 1941) or *Leuconostoc mesenteroides* (Johnson, 1945; Krehl, de la Huerga, Elvehjem & Hant, 1946) including some strains of A.T.C.C. 9135 proved unsatisfactory, as all strains of both types of bacteria so far available responded to nicotinamide as well as nicotinic acid. It has been decided, therefore, to publish the results so far obtained.

Surviving rat liver, but not kidney or muscle, methylate nicotinamide to nicotinamide methochloride. None of the tissues mentioned converts nicotinic acid into either nicotinamide methochloride or trigonelline (Perlzweig, Bernheim & Bernheim, 1943). Ellinger & Coulson (1944) have shown that in man two separate processes are involved in the formation of nicotinamide methochloride from nicotinic acid; these could be affected separately in certain diseases.

The present paper deals with the site and mechanism of the formation of nicotinamide from nicotinic acid by rat tissue. The only other known formation of a simple acid amide in the animal body is that of glutamine from glutamic acid (Krebs, 1935). The nicotinamide formed could only be determined by measuring the nicotinamide methochloride formed from it by liver slices, and the suitability of this method for quantitative measurement had first to be evaluated. Different rat tissues were then tested for their ability to form the amide with or without the addition of potential NH_3 donors such as glutamine or asparagine.

METHODS

Rats of both sexes, weighing 150–400 g., of black and white Lister stock or albinos of own stock, were fed after weaning on a mixed diet (Ellinger, 1946*b*); their daily urinary output of nicotinamide methochloride and their response to injected nicotinamide were studied by the method of Coulson, Ellinger & Holden (1944) in most cases. For experiment, the rats were stunned and killed by bleeding, the various organs to be examined being removed and kept in closed Petri dishes. The method of examination used was similar to that of Perlzweig *et al.* (1943). Pieces of the organs were quickly weighed, cut into slices 0·2–0·4 mm. thick, washed twice in modified Ringer's solution (Krebs & Henseleit, 1932; Krebs, 1933) and suspended in weighing bottles in 10 ml. of a similar solution containing 1 mg. of ammonium nicotinate. This solution which had previously been saturated with a mixture of O_2 (95 vols.) and CO_2 (5 vols.) was again aerated with the same gas mixture, the bottle closed securely and agitated for 3 hr. at 37°. In some experiments, slices of the organ to be examined were shaken together with liver slices, in others they were shaken separately with the ammonium nicotinate solution, the solution being decanted into another weighing bottle and liver slices added to it. After renewed aeration with the gas mixture, agitation was continued at 37° for 3 hr. In each instance the following controls were carried out. To measure the methylating efficiency of the liver used, 1 mg. nicotinamide was added instead of ammonium nicotinate to liver slices. Blanks without addition of nicotinate or nicotinamide were run, and the nicotinamide methochloride values obtained were subtracted from those of the nicotinate experiments. After shaking, the supernatant liquid was decanted into calibrated centrifuge tubes, the tissue was washed twice with 0·5 ml. of Ringer's solution, the washings being added to the supernatant liquid; 0·5 ml. of 20% (w/v) trichloroacetic acid was added, and after thorough mixing the tubes were kept in a water bath at 75–80° for 30 min. After cooling, the volume was adjusted to 12 ml. with Ringer's solution and the precipitated proteins were removed by centrifugation. In portions of the supernatant liquid nicotinamide methochloride was first estimated by the method of Perlzweig *et al.* (1943) without adsorption on Decalso and by the method of Coulson *et al.* (1944). The former frequently gave erroneously high values since the carbinols, the fluorescence of which was to be measured, were immediately oxidized into pyridones which possess much greater fluorescence efficiency than the carbinols. This method was therefore discontinued.

RESULTS

To test the suitability of the method for the quantitative estimation of nicotinamide, the following experiments were carried out. Results are described in terms of Q_{NM} defined as mg. nicotinamide methochloride formed/g. of tissue/hr.

Table 1 shows that the methylating efficiency of livers of various rats differs considerably, and that this efficiency parallels the height of nicotinamide methochloride elimination of the rat while alive. Different pieces of liver of the same rat, however, show a fairly consistent methylating efficiency, the maximal deviation being less than 10% when pieces of similar size are used. From this it is evident that the amount of nicotinamide present in a solution can be assayed when the methylating efficiency of the same liver is determined with known amounts of nicotinamide.

Table 1. *Methylating efficiency* (Q_{NM}) *of different pieces of the same rat liver and relation of such efficiency to the previous nicotinamide methochloride* (NM) *elimination of the rat when alive*

(Incubation 3 hr. in 5 ml. Ringer's solution with 1 mg. nicotinamide. Each mean is the result of eight experiments with liver slices weighing between 300 and 600 mg.)

No. of rat	One week's average daily NM output (μg./day)	Mean wet wt. of liver slices used (mg.)	Mean NM formed (μg.)	Mean Q_{NM}
1	57	452 S.D. ± 34·9	5·84 S.D. ± 0·264	4·4 S.D. ± 0·47
2	138	459 S.D. ± 36·9	15·55 S.D. ± 0·902	11·4 S.D. ± 0·71
3	417	438 S.D. ± 32·9	35·6 S.D. ± 2·368	27·0 S.D. ± 0·72
4	937	437 S.D. ± 34·8	68·6 S.D. ± 4·634	52·5 S.D. ± 1·31

Table 2 demonstrates that if the amount of inger's solution, of nicotinamide and the time of cubation are kept constant, the Q_{NM} depends on the amount of liver tissue used in the experiment, being greater with small amounts of liver and smaller with larger amounts. Between about 250 and 550 mg., the Q_{NM} is sufficiently constant to obtain comparable results.

able 2. *Dependence of* Q_{NM} *of liver slices on the amount of liver used in the experiment*

(Incubation 3 hr. in 10 ml. Ringer's solution with 1 mg. icotinamide.)

No. of rat	One week's average daily NM output (µg./day)	Wet wt. of liver used (mg.)	NM formed in 3 hr. (µg.)	Q_{NM}
5	228	53	3·6	20·4
		108	6·1	18·7
		158	8·7	18·3
		217	11·2	17·2
		263	13·3	16·8
		302	14·7	16·2
		357	17·9	16·7
		417	20·9	15·9
		480	23·5	16·3
		550	25·5	15·4
		627	29·0	15·2
		754	31·9	14·1
		877	33·6	12·8
		1012	36·9	12·2

Two more similar experiments yielded essentially identical results.

The speed of methylation was examined by incubation of pieces of the same liver (300–400 mg.) with 1 mg. nicotinamide for 0·5, 1, 2, 3 and 4 hr. and measuring the methylated product formed. The speed was fairly constant for the first 3 hr. and subsequently slowed down. An incubation of 3 hr. was therefore adopted. The influence of nicotinamide concentration on the Q_{NM} was measured by varying the amounts of nicotinamide added between 50 µg. and 5 mg. Under the conditions of the experiment, amounts increasing from 50 to 200 µg. produced linearly increasing amounts of the methylated product, but between 200 and 500 µg. a maximum of methylation was reached. The effect of pH on the methylating mechanism was examined by varying the pH (measured by glass electrode before incubation) of the Ringer's solution. The pH was varied by changing the bicarbonate concentration. Table 3 shows that the methylation of nicotinamide by liver depends on the pH and has a maximum between pH 7·35 and 7·18.

The effect of anaerobiosis on the methylating mechanism of the liver was examined either by saturating Ringer's solution with a mixture of N_2 (95 vol.) and CO_2 (5 vol.) or by adding KCN (10^{-5}, 10^{-4} or 10^{-3} M) to the Ringer's solution. Table 4 demonstrates that the methylating process is strictly aerobic and inhibited by cyanide. To examine the influence on the Q_{NM} of the thickness of the slices, these were cut 0·2–0·4 and 1–2 mm. thick from pieces of 500–550 mg. of the same liver and incubated under identical conditions. The approximate thickness was determined by cutting cubes from liver, measuring their length, and dividing by the number of slices obtained. No appreciable difference could be seen when slices of 0·2, 0·3 and 0·4 mm. were used, but slices of 1 or 2 mm. were 10–20 % less efficient.

Table 3. *Influence of* pH *on methylation of nicotinamide by liver slices*

(Incubation 3 hr., 1 mg. nicotinamide.)

No. of rat	Concentration of bicarbonate (M)	pH of Ringer before incubation	Wet wt. of liver used (mg.)	NM formed (µg.)	Q_{NM}	Percentage of maximum
14	0·130	8·23	371	9·4	8·4	48·1
	0·100	8·10	358	11·5	10·7	61·0
	0·070	7·87	367	14·6	13·2	75·1
	0·040	7·69	382	19·0	16·6	94·9
	0·020	7·35	353	18·3	17·3	98·8
	0·010	7·18	388	20·4	17·5	100·0
	0·005	6·76	367	15·7	14·3	81·5
	0·002	6·41	359	11·6	10·8	61·6

One other experiment yielded essentially identical results.

Table 4. *Influence of anaerobiosis and cyanide on the methylation of nicotinamide by liver slices*

(Incubation 3 hr., 1 mg. nicotinamide.)

No. of rat	Gas mixture	Concentration of KCN (M)	Wet wt. of liver used (mg.)	NM formed (µg.)	Q_{NM}
16	O_2/CO_2	0	396	29·4	24·7
	O_2/CO_2	0	363	27·4	25·1
	N_2/CO_2	0	372	2·2	0·2
	N_2/CO_2	0	388	0	0
	O_2/CO_2	10^{-5}	352	20·9	19·8
	O_2/CO_2	10^{-4}	393	11·9	10·1
	O_2/CO_2	10^{-3}	376	3·1	2·7

The livers used in the methionine experiment (Table 5) were chosen from various rats, whose individual spontaneous nicotinamide methochloride elimination and whose response to injected nicotinamide had been found to differ markedly. The addition of methionine to the system nicotinamide, liver, Ringer's solution, had in many cases no effect on the methylating efficiency. However, in some instances, particularly when the initial methylating efficiency was low, methionine increased the Q_{NM} appreciably.

Table 5. *Influence of added methionine on the methylation of nicotinamide by liver slices*

(Incubation 3 hr., 1 mg. nicotinamide.)

No. of rat	One week's average daily NM output (mg./day)	Methionine (mg.)	Wet wt. of liver used (mg.)	NM formed (µg.)	Q_{NM}
1	57	0	443	5·6	4·2
		0	491	6·6	4·5
		1	455	10·7	7·8
		1	477	11·5	8·0
2	138	0	437	14·8	11·3
		0	485	16·8	11·5
		1	422	16·6	13·1
		1	447	17·3	12·9
3	417	0	412	34·0	26·8
		0	453	36·9	27·1
		1	423	33·3	26·2
		1	437	34·8	26·6
4	937	0	402	64·6	53·4
		0	449	71·0	52·8
		1	438	68·9	52·5
		1	453	72·8	53·6

Three more experiments yielded essentially identical results.

Liver alone synthesized nicotinamide methochloride from nicotinamide (Tables 6–8), but not from nicotinic acid (Tables 6, 7). If, however, glutamine was added to the system liver, ammonium nicotinate, Ringer's solution, some formation of nicotinamide methochloride could be observed in about half of the experiments, the addition of asparagine having no effect (Table 6). The glutamine used was tested before use with Nessler's reagent (Archibald, 1944).

Table 6. *Influence of added glutamine* (Gl) *or asparagine* (As) *on the formation of nicotinamide methochloride* (NM) *from* 1 *mg. of ammonium nicotinate* (Am.nic.) *by liver slices*

(Incubation 3 hr.)

No. of rat	Added Gl or As (mg.)	Substrate	Wet wt. of liver slice (mg.)	NM formed (µg.)	Q_{NM}
22	0	Nicotinamide	433	24·5	18·8
	0	Nicotinamide	448	25·9	19·3
	0	Am.nic.	471	0·3	0·2
	0	Am.nic.	406	0	0
	10 Gl	Am.nic.	423	0	0
	10 Gl	Am.nic.	434	3·0	2·3
	10 Gl	Am.nic.	444	11·1	8·3
	10 Gl	Am.nic.	412	11·9	9·6
	10 As	Am.nic.	433	0·1	0·1
	10 As	Am.nic.	428	0·4	0·3

Four more experiments yielded essentially identical results.

The following organs were tested for amidation of nicotinic acid: kidney cortex, brain cortex, spleen,

Table 7. *Synthesis of nicotinamide methochloride* (NM) *from ammonium nicotinate* (Am.nic.) *and nicotinamide* (NA) *by slices of various organs*

(Incubation 3 hr.)

Tissue	No. of experiments	Limits of wt. of liver (mg.)	Substrate	Limits of wt. of tissue other than liver (mg.)	NM formed/g. liver/hr. (µg.) (average in brackets)
Liver	43	230–610	NA, 1 mg.	—	3·6–73·5 (27·3)
Liver	22	290–580	Am.nic., 1 mg.	—	0–2·9 (0·5)
Kidney	7	—	NA, 1 mg.	230–320	0–2·1 (0·5)
Kidney	7	—	Am.nic., 1 mg.	220–340	0–1·2 (0·3)
Brain	8	—	NA, 1 mg.	180–280	0–1·9 (0·3)
Brain	8	—	Am.nic., 1 mg.	170–300	0–0·8 (0·2)
Liver	7	354–560	Kidney + NA, 1 mg.	140–590	4·3–58·2 (26·4)
Liver	30	340–590	Kidney + Am.nic., 1 mg.	255–585	4·1–62·3 (22·1)
Liver	8	360–580	Kidney + Am.nic., 1 mg. (anaerobically)	330–470	0–1·3 (0·3)
Liver	7	360–440	Brain + NA, 1 mg.	130–190	7·2–59·2 (26·1)
Liver	20	380–520	Brain + Am.nic., 1 mg.	120–210	7·6–51·3 (21·7)
Liver	4	365–490	Brain + Am.nic., 1 mg. (anaerobically)	140–190	0–1·1 (0·3)
Liver	4	330–430	Spleen + Am.nic., 1 mg.	165–225	0
Liver	4	320–480	Skelet. muscle + Am.nic., 1 mg.	530–620	0
Liver	4	330–450	Heart muscle + Am.nic., 1 mg.	260–300	0
Liver	4	350–450	Pancreas + Am.nic., 1 mg.	50–70	0–0·9 (0·3)
Liver	4	380–510	Intestin. mucous membrane + Am.nic., 1 mg.	110–130	0–0·8 (0·2)

skeletal muscle, heart muscle, pancreas and intestinal mucosa. In each case slices were incubated with 1 mg. of ammonium nicotinate and liver slices, and parallel experiments were made with nicotinamide instead of nicotinate. In addition, the formation of nicotinamide methochloride from ammonium nicotinate or nicotinamide by kidney or brain slices without liver was tested. Table 7 shows that, of all tissues tested, only kidney and brain cortex were able to form nicotinamide from ammonium nicotinate. In some of the experiments, the pyridine carbinols were immediately oxidized to pyridones when estimated by the method of Coulson *et al.* (1944) when kidney was agitated together with liver. No measurement was possible owing to the different fluorescence efficiencies of the

Table 8. *The influence of disintegration of liver tissue on the methylation of nicotinamide and of kidney and brain on the amidation of ammonium nicotinate* (Am.nic.)

(Incubation 3 hr.)

No. of rat	Tissue	Wet wt. of liver used (mg.)	Substrate	NM formed (μg.)	NM formed/g. liver/hr. (μg.)
27	Liver slices	354	1 mg. NA	24·8	23·3
	Liver slices	377	1 mg. NA	26·8	23·7
	Liver broken up cells	348	1 mg. NA	0·2	0·2
	Liver broken up cells	386	1 mg. NA	0	0
	Liver slices	349	1 mg. Am.nic.—302 mg. kidney slices	18·6	17·8
	Liver slices	362	1 mg. Am.nic.—312 mg. kidney slices	19·8	18·2
	Liver slices	380	1 mg. Am.nic.—331 mg. kidney broken up cells	0·4	0·3
	Liver slices	337	1 mg. Am.nic.—347 mg. kidney broken up cells	0·1	0·1
	Liver slices	367	1 mg. Am.nic.—173 mg. brain slices	17·8	16·2
	Liver slices	358	1 mg. Am.nic.—194 mg. brain slices	18·8	17·4
	Liver slices	371	1 mg. Am.nic.—167 mg. brain broken up cells	0·1	0·1
	Liver slices	349	1 mg. Am.nic.—203 mg. brain broken up cells	0	0

Table 9. *Influence of* pH *on amide formation from ammonium nicotinate by kidney and brain slices*

(Incubation 3 hr., 1 mg. ammonium nicotinate.)

No. of rat	Concentration of bicarbonate (M)	pH of solution before incubation	Wet wt. of liver (mg.)	Wet wt. of kidney (K.) or brain (B.) (mg.)		NM formed (μg.)	Q_{NM}	Percentage of maximum
28	0·130	8·23	356	259	K.	8·4	7·9	37·2
	0·100	8·10	349	254		11·3	10·8	51·0
	0·070	7·87	377	263		16·1	14·3	67·4
	0·040	7·69	383	258		20·8	18·3	86·0
	0·020	7·35	354	255		22·0	20·7	97·3
	0·010	7·18	392	260		25·1	21·3	100·0
	0·005	6·76	351	257		22·7	19·8	93·2
	0·002	6·41	366	256		15·1	13·7	64·5
	0·130	8·23	348	202	B.	7·1	6·8	30·1
	0·100	8·10	352	207		10·0	9·5	49·4
	0·070	7·87	361	203		13·8	12·7	66·0
	0·040	7·69	354	208		17·0	16·1	83·7
	0·020	7·35	357	204		20·2	18·9	98·0
	0·010	7·18	349	203		20·2	19·3	100·0
	0·005	6·76	360	207		19·5	17·7	92·8
	0·002	6·41	352	202		13·6	12·9	67·0

two compounds and these experiments had to be discarded. The mechanism of amidation by kidney and brain was studied under anaerobic conditions by using a mixture of N_2 (95 vol.) and CO_2 (5 vol.) for saturating and aerating Ringer's solution in which the kidney or brain were incubated. Before adding the supernatant liquid to the liver slices, it was thoroughly aerated with the O_2-CO_2 mixture for 15 min. The methylating process was strictly aerobic.

Broken up cells (*brei*) of liver, kidney and brain were also tested for their amide-forming and methylating activities. They were prepared by grinding the finely cut tissue in an agate mortar. Table 8 shows that the methylating effect of the liver as well as the amidation by kidney or brain is bound up with the intact structure of the cell.

To investigate the dependence on pH of the amidation by kidney or brain, slices of these organs were incubated in Ringer's solutions of various pH (glass electrode) prepared by using varying amounts of bicarbonate. In order, however, to avoid an effect of altered pH on the methylation by liver, the supernatant liquid after incubation was adjusted to pH 7·2 by addition of bicarbonate or hydrochloric acid solutions (external indicators) before it was incubated with the liver slices. The amounts of kidney or brain used in this experiment differed from each other by less than 10%. Table 9 shows that the amide formation by kidney and brain depends on the pH. The maximum in both cases lies between pH 7·35 and 6·76.

DISCUSSION

Examination of the conditions under which liver slices methylated nicotinamide showed the reaction to be suitable as a qualitative test for nicotinamide and, under strictly controlled experimental conditions, also to provide a quantitative measure of small amounts of nicotinamide in the presence of nicotinic acid. The findings of Perlzweig *et al.* (1943) were confirmed that liver, but neither kidney nor muscle, methylated nicotinamide, that methionine increased the methylation in some cases, that methylation proceeded at a fairly constant speed for about 3 hr., that a maximum effect was reached by amounts of 250 μg. nicotinamide/350–400 mg. of liver, that the process is aerobic and requires intact cells; but, contrary to the findings of Perlzweig *et al.* (1943), it was found that different pieces of liver of the same rat were of the same methylating efficiency. Perhaps their 'somewhat erratic results' are due to their method of assay without adsorption and elution. Some information could be obtained concerning properties of the enzymic system responsible for nicotinamide methylation such as dependence on pH, inhibition by anaerobic conditions and HCN, and dependence on intact cell structure. The specificity of the enzyme for nicotinamide is demonstrated by the observation of Perlzweig *et al.* (1943) that after incubation of nicotinic acid with liver slices no trigonelline formation was observed with the method of Sarett (1943), and by Ellinger, Fraenkel & Abdel Kader's findings (1947) that none of a large number of nicotinamide derivatives was methylated by the enzyme.

Of all organs tested for their ability to convert nicotinic acid into nicotinamide, only kidney and brain were found active. These are the same organs which were shown by Krebs (1935) to form glutamine from ammonia and glutamic acid; retina was not examined in the present experiments. A few properties of the nicotinic acid amidase were investigated, such as pH activity curve and dependence on the presence of oxygen and intact cell structure. The specificity could not be tested; this can only be done when an exact method for separate estimation of nicotinamide and nicotinic acid becomes available.

Certain difficulty attended the construction of a pH activity curve for the nicotinic acid amidase, since the nicotinamide formed could be measured only indirectly by means of two separate biological processes with all their attendant inaccuracies. Unnecessary corrections were eliminated as far as possible by using constant amounts of kidney and brain.

No explanation can be given at present for the peculiar effect of glutamine on the conversion of nicotinic acid into nicotinamide methochloride by liver tissue, or for the finding that this effect occurs only on some occasions.

SUMMARY

1. The methylation of nicotinamide by liver tissue *in vitro* has been studied and found to be suitable as a qualitative, and with certain restrictions as a quantitative assay method for nicotinamide in the presence of nicotinic acid. The conditions of this process have been examined and some properties described of the enzyme system involved.

2. Kidney and brain have been found to convert nicotinic acid into nicotinamide *in vitro*. Some of the properties of the enzyme system involved in this amide formation have been studied.

3. Liver did not convert nicotinic acid into nicotinamide methochloride *in vitro*, but when glutamine was added formation of nicotinamide methochloride from nicotinic acid occurred in some instances.

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Electrophoretic Studies on Human Serum

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The purpose of the present work was to collect information of the extent to which the interpretation of electrophoretic patterns of serum proteins in phosphate buffer can account for 'boundary anomalies', an expression used by Tiselius (1930) and others to refer to small changes in field intensity and mobility across a boundary.

Although the nature of the changes in conductivity and ionic mobility across a protein boundary has been recognized for some time (Tiselius, 1930; Longsworth & MacInnes, 1940*a*; Abramson, Moyer & Gorin, 1942), it is only recently that these changes have been given a detailed theoretical consideration (Svensson, 1943; Dole, 1945; Svensson, 1946). A theoretical treatment is here given which incorporates Svensson's conclusions on boundary anomalies and has been used to study in particular (*a*) the relative migration velocities of the α_2 and A boundaries in human serum, and (*b*) the conditions during electrophoresis of solutions containing protein components of nearly the same mobilities.

THEORETICAL

Notation

The symbols used are defined as follows:

V_i = migration of protein i (numbered from the fastest component) at infinite dilution in the buffer of concentration equal to that immediately below the ϵ boundary;

V_{ai} = actual migration velocity of protein i below its boundary in descending limb;

r = ratio of velocities of the protein ions at infinite dilution in the bulk buffer to that in buffer below ϵ boundary;

C_i = apparent concentration of protein i on descending side $= \dfrac{\text{area under peak } i}{\text{total area desc.} \ -\epsilon} \times$ concentration of protein in starting solution;

C_1' = apparent concentration of protein i on ascending side $= \dfrac{\text{area under peak } i}{\text{total area asc.} \ -\delta} \times \rho \times$ concentration of protein in starting solution;

C_i and C_1' are expressed in g./100 ml.;

$$\rho = \frac{\text{mean of asc. and desc. total areas} \ -\delta}{\text{mean of asc. and desc. total areas} \ -\epsilon};$$

a_i, a_i' = boundary displacements on the descending and ascending sides during an arbitrary time;

K_i = coefficients as defined in the text;

κ = conductivity;

I = current per unit area of cross-section.

Notation for Svensson's (1946) *equation* (23)

c_{i1} = ionic concentrations in electrochemical equiv./ml. above colloid boundary, with the signs of the charges;

u_{i1} = ionic mobilities in cm.^{2} V.$^{-1}$ sec.$^{-1}$ above colloid boundary, with the signs of the charges;

u_2/u_1 = mobility ratio across the colloid boundary;

C = concentration of a leading ion in equiv./ml. with the sign of the charge;

$\mathbf{U}$ = mobility of colloid ion above the boundary, defined by equation (22) of Svensson (1946); $\mathbf{U} = \overline{\mathrm{U}}$ of Svensson;

κ_2/κ_1 = ratio of conductivities of solutions above and below the colloid boundary.

The displacement of the centroidal ordinate of the gradient curve of a boundary gives the migration velocity of the protein ion in the environment below its boundary (Longsworth, 1943). If the relative change in migration velocity of a protein ion, brought about by a change in the concentration of protein from zero to C_i is put proportional to the protein concentration, i.e. $(V_{ai}-V_i)/V_i=K_iC_i$, then the velocity of, for example, the second slowest descending boundary is given by $V_{i-1}(1+K_iC_i+K_{i-1}C_{i-1})$, that of the fastest ascending boundary by $rV_1(1+K_1C'_1)$. Generally:

$$\begin{aligned}&V_1(1+K_iC_i+K_{i-1}C_{i-1}+\ldots+K_3C_3+K_2C_2+K_1C_1):\\&V_2(1+K_iC_i+K_{i-1}C_{i-1}+\ldots+K_3C_3+K_2C_2):\ldots:\\&V_i(1+K_iC_i):rV_1(1+K_1C_1'):rV_2(1+K_1C_1'+K_2C_2'):\ldots:\\&rV_i(1+K_1C_1'+K_2C_2'+\ldots+K_iC_i')\\&\qquad=a_1:a_2:\ldots:a_i:a_1':a_2':\ldots:a_i'.\end{aligned}\tag{1}$$

The coefficients K_i are intended to account for both the increased field strength and the changed mobility below the protein boundary.

The conditions necessary for these simple proportionality relations to hold can be derived from the equation for the conductivity change across a boundary given by equation (23) of Svensson (1946):

$$\frac{\kappa_2}{\kappa_1}=\frac{u_2}{u_1}\left[1-\frac{C}{\Sigma\dfrac{c_{i1}u_{i1}}{u_{i1}-\mathbf{U}}}\right].$$

This implies

$$-\frac{\Delta\kappa}{\kappa_1}+\frac{\Delta u}{u_1}=\frac{u_2}{u_1}\,\frac{C}{\Sigma\dfrac{c_{i1}u_{i1}}{u_{i1}-\mathbf{U}}}.\tag{2}$$

However,

$$-\frac{\Delta\kappa}{\kappa_1}+\frac{\Delta u}{u_1}=\frac{(V_{ai}-V_i)\kappa_2}{V_i\kappa_1}$$

for a protein i, where

$$V_{ai}=\frac{u_{i2}I}{\kappa_2}\quad\text{and}\quad V_i=\frac{u_{i1}I}{\kappa_1},\tag{3}$$

u_{i2}/u_{i1} of the protein ions being taken equal to u_2/u_1. After elimination of κ_2/κ_1,

$$\frac{V_{ai}-V_i}{V_i}=\frac{C}{\Sigma\dfrac{c_{i1}u_{i1}}{u_{i1}-\mathbf{U}}-C}.\tag{4}$$

The conditions for equation (1) to hold are then (*a*) that C be small compared with $\Sigma\dfrac{c_{i1}u_{i1}}{u_{i1}-\mathbf{U}}$, $K_i=\dfrac{1}{V_i}\,\dfrac{V_{ai}-V_i}{C_i}$ being then an approximation to $\left(\dfrac{1}{V_i}\dfrac{dV_{ai}}{dC_i}\right)_{C_i\to 0}$ at constant buffer salt concentration in the supernatant fluid, and (*b*) that u_{i2}/u_{i1} for the protein ions be approximately equal to u_2/u_1 for the buffer ions. It is considered that these conditions are satisfied in the experiments described in the present paper on account of the small specific charge of the protein ions, and since the changes in pH across the protein boundaries could be assumed to be negligible. If other protein components are present on both sides of the protein boundary, the V_i refer to the velocity in buffer in the absence of the other protein ions as stated in the definition. The use in equation (1) of the apparent concentrations, where strictly the true concentrations are concerned, was taken as an approximation for cases in which the concentrations of the protein components on both sides of protein boundaries could be assumed to differ little. When two components have very similar mobilities, the true concentrations must be used in the calculation, as is done in a later section of this paper. Equation (1) has been derived for a special purpose for which an extended treatment was not considered necessary, the advantages conferred by simplicity outweighing the limitations due to the imposed conditions.

EXPERIMENTAL

The electrophoresis experiments were carried out in the Tiselius apparatus fitted with the Philpot-Svensson diagonal edge optical system. In place of the diagonal edge a wire of 0·19 mm. diameter was used. The technique of electrophoresis has been described by several authors (see Svensson, 1946; Longsworth, 1946; Wiedemann, 1946). As can be seen from the base lines in the accompanying photographs, the optical system used has not the perfection of spherical correction attainable by more recent developments. It was tested for inadequacies not obvious in the appearances of the base lines by measuring the areas given by salt boundaries which had been moved to four different positions on each limb, and it was found that the range of variation in the values for the areas (±1·5%) did not appreciably exceed the experimental error. The optical system was also tested by comparing the patterns given by a lens (one lens of focal length 200 cm. and another of focal length 25 cm.) placed at different positions in a vertical plane at the place of the **U**-tube. A mm. scale, placed between the limbs of the **U**-tube, gave a practically undistorted image (without the cylindrical lens; within ±0·05 mm. in all and within ±0·03, mm. in 31 out of 34 pairs of measurements).

Serum was diluted with phosphate buffer of pH 8·0 and ionic strength $\mu=0{\cdot}1$, and dialyzed against 2 l. of buffer in the refrigerator at 0–4° for several days with frequent mixing. The **U**-tube was of the medium-size type requiring about 10·5 ml. to fill the bottom compartments and the bridge. Currents between 3 and 20 mA. were used (cross-section of the **U**-tube = 75 sq.mm.). The bath temperature was 0·1°. The base lines were photographed before each experiment was started, after the bottom compartment had been set in alignment. The areas were measured with a planimeter on enlarged tracings. The method of dividing the areas was that of Svedberg & Pedersen (1940) as used by Longsworth (1946). For grossly asymmetric curves, like those of the β and γ peaks, curves of different steepness were used for the extrapolation of each side. The small component behind the β-globulin was always included in the γ-globulin. The displacements of the albumin and the α-globulin boundaries only were measured, since the position of the β-globulin boundary on the descending side is disturbed by the presence of the β-anomaly, and the relative migration velocity of the γ peak is too small for the accuracy of measurement required. The area of the β-globulins, however, can be obtained correctly from records taken 10–120 min. after the current has been stopped, when the anomalous part of the curve has shrunk to within the measurable range. Any material flowing off or collecting in the β boundary should, according to Moore & Lynn (quoted by Abramson *et al.* 1942, p. 188), not alter the value for the area underneath the β-globulin peak. The difference in the displacements of the A and α boundaries was determined from measurements of the centroidal ordinates of the gradient curves (Longsworth, 1943; Dole, 1945) to an accuracy of 0·3 mm. or better. Ordinates at intervals of 0·2 mm. were placed parallel to the streaks on the pattern (originating from dust particles in focus on the plate), and the position of the centroidal ordinate was obtained by $\mathbf{x}=\sum_i x_i y_i / \sum_i y_i$, where x_i is the distance in the direction of migration and y_i that along the ordinate. While it was possible to locate the *A* boundaries in the descending limb to within ±0·1 mm. or better, the location of the α boundary was often made more uncertain since an appreciable part of the curve was obtained by extrapolation. The centroidal ordinates for the ascending albumin boundaries at concentrations higher than 1 g./100 ml. were obtained by extrapolating the top part of the gradient curve which fell outside the range covered by the photographic record. The area of this part of the peak was calculated by difference of the total area of the ascending *A* peak and that of the available part, the total area of the ascending *A* peak from that of the descending *A* peak by multiplication by ρ, and ρ was obtained as defined above assuming the total areas under all peaks (including δ and ϵ) to be equal on both sides. All these values are crude approximations, but errors in neither of them affect the position of the centroidal ordinate significantly. If the missing part of the curve is taken to be symmetrical with respect to an ordinate half way between the highest (equal) ordinates measured, then the same value for the centroidal ordinate is obtained, independent of the shape of this part of the curve, which may be between that of an equilateral triangle and a rectangle. The most probable shape of the missing part of an asymmetrical curve will be between that of a rectangle and that of a triangle, the sides of which are in the same ratio as the areas under the curve outside the highest ordinates measured, but nearer that of the latter. The differences in the positions of the centroidal ordinates for the extreme cases were in the present experiments between 0·016 and 0·033 mm. The position of the centroidal ordinate used in the calculation for Exps. 3 and 7–10 of Table 1 was that calculated for the missing part of the curve as an asymmetrical triangle.

The displacements were measured from the position of the original boundary, and no correction was applied for volume changes due to the electrode reactions (Longsworth, 1943; Dole, 1945).

RESULTS

The relative migration velocities of the α_2 and A boundaries

For a system of four components such as that of the four groups of proteins in human serum (phosphate buffer) the appropriate equations are:

$$V_1\,(1+K_4C_4+K_3C_3+K_2C_2+K_1C_1):$$
$$V_2\,(1+K_4C_4+K_3C_3+K_2C_2):rV_1\,(1+K_1C_1'):$$
$$:rV_2\,(1+K_1C_1'+K_2C_2')=a_1:a_2:a_1':a_2'. \quad (5)$$

They can be solved for r and the K_i if all concentrations and displacements are known and if a relationship is assumed between the coefficients K_i. For the present purpose the values of K_1 and r were calculated from (5) by assuming K_4, K_3 and K_2 to be equal to K_1. The mean values from both patterns of the apparent relative concentrations (assuming, as is usual, equal refractive indices for all components), multiplied by the protein concentration of the starting solution, were taken as the values for the apparent concentrations on the descending side. The concentrations of albumin and α_2-globulin on the ascending side were calculated from the mean values by multiplication by ρ as the best approximation to the values of the apparent concentrations. Where the area of the albumin peak on the ascending side could not be measured, the areas on the descending pattern only were used for calculation. The area of ϵ, which could in no serum pattern be defined with any certainty, was taken as one fifth of δ. The ratio ϵ/δ for the equilibrated solution of albumin in Fig. 1 A and B was found to be $0{\cdot}2_7$. On account of the nature of equation (5) there was no necessity for any great accuracy in the location of the position of the original boundary, provided all measurements on both sides were taken from points equidistant from the centre line between the partition plates. In order to make use of patterns where no record of the position of the original boundary had been taken, values for K_1 and r were bracketed within the limits obtained by the use of the distances of the boundaries from the δ and the descending γ boundaries respectively.

Table 1 shows the results obtained from ten electrophoresis experiments on normal and pathological sera. The values for r incorporate the ratio of the cross-sections of both sides of the **U**-tube, for which no correction has been applied. They are, furthermore, much dependent on the assumed relationship between the K_i and they do not, therefore, represent an experimental approach to obtaining the ratio of velocities as stated in their definition, but merely an aid for obtaining values for K_i, in the calculation of which they are made to cancel out.

The coefficient K_1 was about 0·1 in all experiments, signifying an increase of about 10 % in the migration velocity for an increase of 1 g./100 ml. in the protein concentration. Such a high value was rather unexpected, and therefore the effects on K_1 of the uncertainty involved in the values employed for ρ and δ were tested by repeating the calculation, putting in their place $\frac{1}{2}(\rho+1)$ or $\frac{1}{2}\delta$. (The altered value of ρ was used with the same relative concentrations of the components, while that of δ involved a change in the relative concentrations, mainly in that of γ-globulin.) For example, using the value $\frac{1}{2}(\rho+1)$, K_1 in Exp. 3 was then found to be lowered by 0·001. Using $\frac{1}{2}\delta$ in Exp. 2 the same value for K_1 was found. By taking $K_4=K_3=K_2=0$ the value for K_1 in Exp. 10 was increased by 0·0009. By taking $K_4=K_3=K_2=2K_1$ the value for K_1 was 0·0026 lower; that for K_2, etc., worked out to 0·19, which is improbably high. Thus a drastic change in the assumptions about the coefficients or errors in the estimates of ρ or δ did not alter K_1 to any considerable extent.

Another estimate of K_1, not involving proteins other than albumin, was therefore obtained by comparing the distances migrated by the ascending albumin boundaries at different concentrations of serum. Serum was diluted to a total protein content of 0·61, 1·17 and 2·38 g./100 ml., and all three samples dialyzed together against 2 l. of buffer. The three electrophoresis experiments on them (Exps. 1, 2 and 3 of Table 1) were carried out on one day against the same supernatant fluid and without change of the liquids in the electrode vessels.

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Table 1. *Coefficient K_1 derived from the relative migration velocities of the α_2 and A boundaries*

Exp. no.	Diagnosis	Conc. of protein (g./100 ml.)	Albumin ($+\alpha_1$) (% of total)	K_1	r
1	Normal	0·6	65	0·21	1·12
2		1·2		0·12	1·17
3		2·4		0·12	1·32
				(δ as origin) ($\gamma_{\text{desc.}}$ as origin)	(δ as origin) ($\gamma_{\text{desc.}}$ as origin)
4	Normal	1·4	62	0·08—0·12	1·13—1·18
5	Normal	1·5	63	0·11—0·15	1·20—1·27
6	Plasma-cell myeloma	1·9	50	0·13—0·17	1·29—1·36
7	Breast carcinoma	2·4	70	0·07—0·11	1·23—1·29
8	Breast carcinoma	2·5	58	0·09—0·12	1·28—1·35
9		2·5	60	0·10—0·14	1·33—1·42
10	Cirrhosis of liver	2·5	57	0·09—0·13	1·30—1·36

Table 2. *Migration velocities of albumin boundaries and apparent composition of serum (in g./100 ml.) at different concentrations*

(Phosphate buffer pH 8·0, $\mu=0{\cdot}1$, 120 min. at 12·0 mA.)

Concentration of total protein (g./100 ml.)	0·61			1·17			2·38			1·4*		
Distances migrated (mm.) Ascending side	$28{\cdot}_2$			$29{\cdot}_3$			$30{\cdot}_0$			—		
Descending side	$26{\cdot}_4$			$26{\cdot}_2$			$25{\cdot}_6$			—		
	Asc.	Desc.	Mean	Asc.	Desc.	Mean	Asc.	Desc.	Mean	Asc.	Desc.	Mean
Albumin ($+\alpha_1$)	4·47	4·35	4·41	4·65	4·37	4·51	—	4·56	—	4·52	4·47	4·50
α_2	0·50	0·57	0·53	0·33	0·50	0·41	—	0·44	—	0·60	0·43	0·54
β	0·92	0·88	0·90	0·84	0·89	0·87	—	0·90	—	0·83	0·95	0·89
γ	1·01	1·10	1·06	1·08	1·14	1·11	—	1·00	—	0·95	0·99	0·97
Albumin ($+\alpha_1$) $+\alpha_2$	4·97	4·92	4·94	4·98	4·87	4·92	—	5·00	—	5·12	4·96	5·04
$\frac{\delta\text{ (or }\epsilon)}{\text{Total area asc.}}$	—	—	—	0·08	—	—	0·16	—	—	0·11	0·055	—

* Veronal buffer pH 8·6 as used by Longsworth, Curtis & Pembroke (1945).

Table 2 shows the distances migrated in equal times at equal currents. They were measured from the position of the original boundary.

The increase on the ascending side in the distance migrated by the albumin boundary is 6% for an increase in the albumin concentration (corrected for dilution in the ascending limb) of about 1 g./100 ml. The coefficient K_1 therefore works out to be 0·06. The results of the area measurements are included to show the effect of the concentration on the apparent relative concentrations of the components. The sum of $A(+\alpha_1)+\alpha_2$ should be used for comparison on account of the uncertainty involved when extrapolating the curve between $A(+\alpha_1)$ and α_2. The change in the apparent concentration of $A(+\alpha_1)+\alpha_2$ for the whole practicable range is about 2% in the descending patterns, but this is here within the experimental error. Perlmann & Kaufman (1945) found a change in the proportion of the albumin to total protein, as calculated from the areas given by human plasma in veronal buffer, from 58·4 to 54·6% as the total protein concentration was changed from 2·5 to 0·94 g./100 ml. (4 to 1·5 mg. N/ml.). The change in this case is about 6·5%.

Simultaneous measurements of velocities at different concentrations. Small differences in migration velocities could be measured more satisfactorily in simultaneous experiments than otherwise; one such experiment is reported here in detail, since the technique leads to a new method of comparing mobilities. The method was based (*a*) on the theoretical consideration that the δ boundary should be completely absent when the starting solution is a solution of a single component obtained from between the ascending boundary and the δ boundary and when the buffer used as supernatant is the same as that into which the protein component has migrated previously (Lagercrantz, 1945), and (*b*) on the assumption that for low concentrations of the protein the changes in buffer-ion concentrations across the ascending boundary are proportional to the protein concentration.

Experimental evidence for (*a*) was obtained during a preparation of serum albumin. An albumin solution (18 ml.) free from α_1 and α_2 was prepared by several electrophoretic runs on undiluted serum dialyzed against veronal buffer (pH 8·6). The pooled samples were then electrophoresed into veronal buffer and they proved to be free from α_2 or α_1. This solution was then dialyzed with frequent stirring against four changes of 2 l. each of phosphate buffer of pH 8·0 during 12 days. Electrophoresis into phosphate buffer gave the patterns as shown in Fig. 1 A and B. Apart from the albumin boundary and a minute component ahead of it, only the δ and ϵ peaks are seen. The area of the δ peak amounted to 16 % of the total area (protein concentration 1·8 g./100 ml.). This electrophoresis was continued for 6·5 hr. at 20 mA., while the ascending boundary was kept in position by steady compensation, and there was no disturbance by convection on the ascending side, whereas on the descending side convection began when the δ boundary had reached the bridge. The albumin solution from the ascending side, which was expected to show no δ effect, was separated. The remainder of the solution was mixed with the solution, dialyzed against phosphate in the ratio 3 : 2 and electrophoresed (with an interruption of 11 hr.) for 12 hr. at 20 mA. The δ effect was now smaller than in the previous run, since a greater part of the starting solution had had the buffer salt concentration reduced during the previous experiment. This run also lasted much beyond the time at which the δ boundary passed the bridge, and thus the concentration in buffer salts on the descending side must have been further reduced, approaching that on the ascending side. The final run was made with a solution consisting of 2 parts of albumin solution from the ascending side, 1 part of the remainder from the bridge and descending side, and a small amount of buffer to make up the volume required. A small δ peak was observed, the area of which indicated a change in refractive index of 1·7 % of that at the descending A boundary.

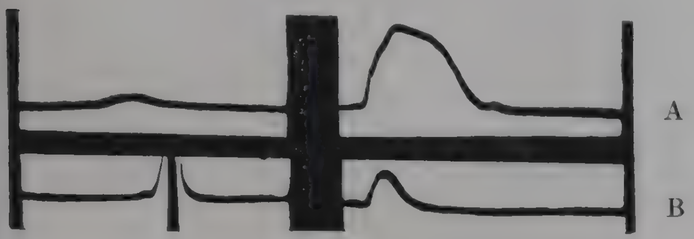

Fig. 1. Electrophoretic patterns of serum albumin free from α_1- and α_2-globulin. A, ascending albumin and δ, after 57 min. at 20 mA., tilt 60° to the horizontal; B, descending albumin and ϵ, after 92 min. at 20 mA., tilt 60°; phosphate buffer pH 8·0, $\mu = 0{\cdot}1$. The direction of migration is to the left for ascending and to the right for descending patterns.

If the assumption that the change in migration velocity at a protein boundary is proportional to the change in protein concentration holds in the range of concentrations used, then no gravitational instability should arise, and no additional δ boundary should appear in the experiment carried out as follows: The layer formed by the electrophoresis of serum between the first two boundaries on the ascending side, A and α_2, was recovered from the **U**-tube, and diluted with buffer to reduce the concentration as much as possible consistent with retaining a distinct boundary peak. This dilute protein solution was then used as supernatant in place of the buffer in the top compartment on the ascending side in the next electrophoresis of the same serum. In both runs portions of the same dialyzed serum and the same buffer against which it has been dialyzed were used. The interference of α_1 can be disregarded, since its concentration in the solution of the diluted albumin is negligible and the difference in mobilities between A and α_1 in phosphate buffer is small.

The difference between the migration velocity of a boundary of a concentrated and that of a diluted albumin solution can be measured directly by the change in the distance between these two boundaries. Table 3 gives the experimental details, and Fig. 2 shows the patterns of an experiment carried out with normal serum. Assuming that the δ boundary has moved 2·5 mm. backward with reference to the position of the original boundary as was observed to be the case in Exp. 3 (Table 1), the distance migrated by the $A_{\text{conc.}}$ boundary in 145 min. was 33 mm. The change in the distance $A_{\text{conc.}} - A_{\text{dil.}}$ was 2·1 mm. and the distance migrated by the $A_{\text{dil.}}$ boundary 30·9 mm. The velocity of the large albumin boundary does not represent the migration velocity of the albumin ions since these ions are present on both sides of the boundary (Svensson, 1946). The difference between the velocity of the boundary, and the true velocity of the albumin ions behind the boundary, can be estimated in the present case in a simple manner on account of the assumption that a change in the concentrations of any ion species cannot occur either behind the $A_{\text{dil.}}$ or between the $A_{\text{conc.}}$ and the α_2 boundary because δ boundary effects, apart from those normally present, are bound to be absent in these regions. Owing to the difference in velocity of the $A_{\text{conc.}}$ and $A_{\text{dil.}}$ boundaries the volume/unit cross section occupied by the dilute albumin solution has been reduced by $rV_1(K_1C'_{\text{conc.}} - K_1C'_{\text{dil.}})$. The amount of albumin in the volume $rV_1(K_1C'_{\text{conc.}} - K_1C'_{\text{dil.}})$ is $rV_1K_1(C'_{\text{conc.}} - C'_{\text{dil.}})\,C'_{\text{dil.}}$. This amount appears in the layer of concentrated albumin, the volume of which (per unit cross section) is increased by

$$\frac{rV_1K_1(C'_{\text{conc.}} - C'_{\text{dil.}})\,C'_{\text{dil.}}}{C'_{\text{conc.}} - C'_{\text{dil.}}}.$$

The length (= volume/unit cross section) of the layer of concentrated albumin is therefore greater by this amount than it would have been if the concentrated

Table 3. *Relative migration velocities of concentrated and diluted serum albumin*

Time (min.)	Current (mA.)	Distances in mm. on plate				Fig.
		$A_{\text{conc.}}-\delta$	$A_{\text{conc.}}-\gamma_{\text{asc.}}$	$A_{\text{desc.}}-\gamma_{\text{desc.}}$	$A_{\text{conc.}}-A_{\text{dil.}}$	
(I) 145	10	$35{\cdot}_5$	$28{\cdot}_4$	$24{\cdot}_4$	—	—
(II) 0	—	—	—	—	$50{\cdot}_9$	A
72	10	—	—	—	$49{\cdot}_9$	B
130	10	—	—	—	$49{\cdot}_2$	C
145	10	$35{\cdot}_5$	$28{\cdot}_4$	$24{\cdot}_4$	$48{\cdot}_8$	—
Further 35	20	(Calculated) $52{\cdot}_6$	—	—	(47·7)	D, F

Normal serum, total protein 6·7 g./100 ml., dialyzed undiluted against 2 l. phosphate buffer (pH 8·0, $\mu=0{\cdot}1$) for 22 hr., diluted with fresh phosphate buffer of pH 8·0, dialyzed against this buffer for 48 hr., after which the protein concentration was 2·02 g./100 ml. (Kjeldahl); electrophoresis (I) for 145 min. at 10 mA., then for 126 min. at 20 mA.; 0·3 ml. of α_2-free albumin from ascending side added to 4 ml. buffer and this solution used as supernatant on the ascending side in the experiment following (II), with the starting solution of diluted serum and buffer the same as in (I); the contents of the electrode vessels were not changed; 10 mA. corresponds to about 5·3 V./cm.; the optical enlarging factor along the direction of the current was 1·12. The composition of the starting solution as calculated from the descending pattern was: $A\,(+\alpha_1)=1{\cdot}34$, $\alpha_2=0{\cdot}11$, $\beta=0{\cdot}30$, $\gamma=0{\cdot}27$ g./100 ml.; the concentration between the A and α_2 boundaries on the ascending side ($C'_{\text{conc.}}$) was approximately $1{\cdot}34\times\rho$, i.e. 1·2; the concentration between the $A_{\text{conc.}}$ and the $A_{\text{dil.}}$ boundaries ($C'_{\text{dil.}}$) was 0·07 g./100 ml. (calculated from the areas).

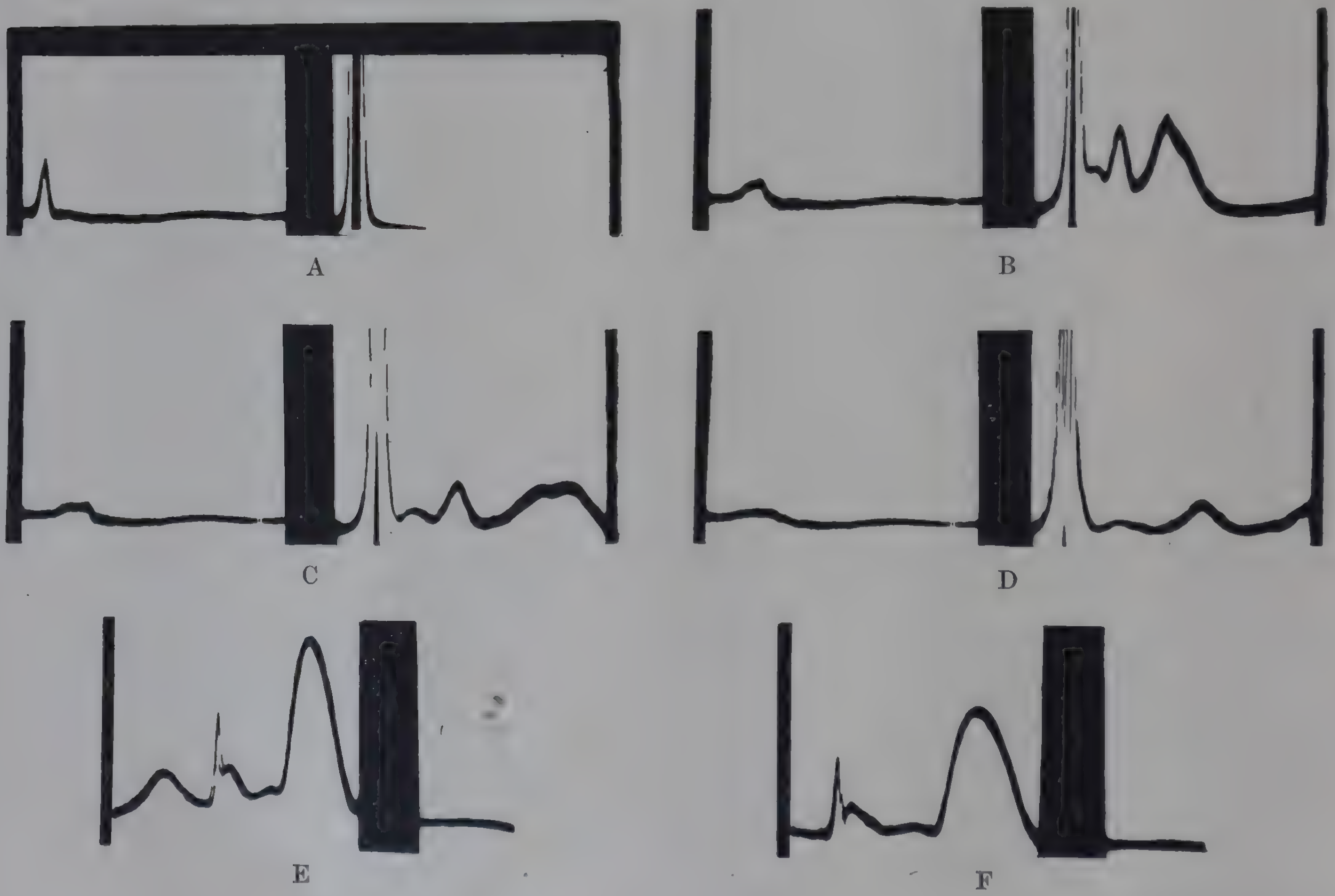

Fig. 2. Migration velocities of the boundaries of dilute and concentrated albumin. A–D, ascending patterns, tilt 45°; A, B and C after 0, 72 and 130 min. at 10 mA., D after 145 min. at 10 mA. and further 35 min. at 20 mA.; E, F, descending patterns, tilt 45°; E, after 145 min. at 10 mA.; F, after further 35 min. at 20 mA. Phosphate buffer pH 8·0, $\mu=0{\cdot}1$.

albumin had migrated into buffer alone. Per unit distance migrated by the $A_{\mathrm{dil.}}$ boundary, this is $\frac{rV_1K_1C'_{\mathrm{dil.}}}{rV_1(1+K_1C'_{\mathrm{dil.}})}$, or very nearly $K_1C'_{\mathrm{dil.}}$. In the present case this is 0·4% of 30·9 mm., or 0·13 mm. This figure has to be subtracted from 2·1 (the change in the distance between the two A boundaries), and the relative increment in velocity/unit concentration, K_1, becomes $\frac{1\cdot97}{30\cdot9\,(1\cdot2-0\cdot07)}$, i.e. 0·056.

The salt concentration in the layer of diluted albumin will be smaller than that which would be obtained after electrophoresis of albumin at this concentration into buffer, by the extent to which K_1 increases as the protein concentration increases. Hence a gravitationally unstable region might be expected to appear behind the $A_{\mathrm{dil.}}$ boundary. This region would sink as soon as it was formed and would appear as an additional peak at the $A_{\mathrm{conc.}}$ boundary. Such disturbances are vanishingly small if the concentration of the dilute albumin solution is below 0·1 g./100 ml.

It should be noted that separation into two components is seen at the boundary of the dilute but not at that of the concentrated solution. This is in accordance with the theory as will be seen in a later section of the paper. Fig. 2 D and F show the patterns after the δ and γ boundaries have reached the bridge. The appearance of the descending pattern has barely changed, since the convections were restricted to the region below the albumin boundary.

The high value for K_1, obtained when calculated from the relative positions of the α_2 and A boundaries in serum patterns, suggests a disturbance in the region of the α_2 boundary, due to inverted density gradients or a mobility ratio for the α_2-globulin across the descending albumin boundary, which deviates considerably from that assumed constant for all ions in Svensson's equation. The velocity of the α_2 boundary relative to that of the A boundary appears too large on the ascending or too small on the descending side or both. The discrepancy in the distance $A-\alpha_2$ is about 0·5 mm. on either the ascending or descending side in a normal pattern of serum diluted to a total protein concentration of 1·5 g./100 ml. (phosphate buffer pH 8·0 and $\mu=0\cdot1$), in which the distance between A and δ is 35 mm. The data given by Lagercrantz (1945) for the corrected mobilities of the components of normal human serum at pH 7·7 and $\mu=0\cdot05$ support the present findings of the anomalous position of the α_2 boundary. The values for α-globulin are 5·57 and $5\cdot06\times10^{-5}$ cm.^{2} V.$^{-1}$ sec.$^{-1}$ for the ascending and descending sides respectively. At $\mu=0\cdot2$, Lagercrantz found agreement between the values for the mobilities of α-globulin.

The net charge of serum albumin. The experimental determination of K_1 makes it possible to calculate the average specific charge of serum albumin at pH 8·0. If C, the equivalent concentration of the colloid ion in Svensson's equation (23) (quoted above), is replaced by $Q_sC_1\times10^{-2}$ (Q_s being the specific charge, expressed as valence/g.) then from equation (4)

$$K_1=\frac{Q_s\times10^{-2}}{\sum\frac{c_{i1}u_{i1}}{u_{i1}-\mathrm{U}}-C}, \tag{4a}$$

and Q_s can be calculated, C being neglected (or used as a correction term after Q_s has been found). The following numerical values were used:

$$c_{\mathrm{Na}^+1}=-c_{\mathrm{HPO_4}^=1}=0\cdot067\times10^{-3}\ \text{equivalents/ml.,*}$$

$$u_{\mathrm{Na}^+1}=2\cdot35,\dagger\quad u_{\mathrm{HPO_4}^=1}=-2\cdot19,\ddagger$$

$\mathrm{U}=-0\cdot7$ (mobilities in 10^{-4} cm.^{2} V.$^{-1}$ sec.$^{-1}$),§ $K_1=0\cdot06$. Q_s then becomes $-2\cdot8\times10^{-4}$. The net charge of human serum albumin (molecular weight 70,000) at 0°, pH 8·0, and $\mu=0\cdot1$ is therefore about -19. Cohn, Oncley, Strong, Hughes & Armstrong (1944) quote -18 as the net charge of human serum albumin at pH 7·4. If the values for horse serum albumin B, calculated by Abramson *et al.* (1942) from a combination of electrophoresis, diffusion, and sedimentation data at 25° and $\mu=0\cdot1$, are extrapolated to a pH of 8·0, values of about -20 are obtained. The extrapolated values for pH 8·0 of the valence, calculated from titration data by the same authors, are about -30 and -21 for $\mu=0\cdot1$ and 0 respectively.

An accuracy of 10% in the value for the net charge as determined here implies an accuracy of 1% in the values for the boundary displacements. Both the corrections for the volume changes in the electrode vessels and those caused by changes in the solvent concentration at the boundary, which were discussed by Dole (1945), seem in this case of minor importance. One advantage of this method is its independence of the starting conditions in cases in which the Donnan equilibrium has not been completely established, since the protein concentration below the boundary can be estimated by chemical methods or from the electrophoretic pattern. But the main merit appears to be that the net charge of a leading ion can be obtained without previous separation from other components.

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* A small error only is introduced by disregarding the presence of $H_2PO_4^-$ (about 10 mol./100) and assuming all phosphate to be in the form of $HPO_4^=$.

† Landolt-Börnstein, *Physikalisch-Chemische Tabellen.*

‡ Landolt-Börnstein, extrapolated to 0° and 0·033 mol./l. from a value given for 25° at infinite dilution, assuming the same relative dependency on temperature and concentration as the $SO_4^=$ ion.

§ Extrapolated from values for different ionic strengths and pH given by Lagercrantz (1945), who derived the values for the mobilities from conductivity measurements on particular sections of the contents of the **U**-tube, and others (Stenhagen, 1938; Longsworth & MacInnes, 1940*b*; Blix, 1941; Kendall, 1941; Chargaff, Ziff & Moore, 1941; Cohn *et al.* 1944; Moore, 1945).

Boundary relationships with two components of similar mobilities

The statement of the conservation of matter applied to the protein ions overtaking an ascending boundary leads, with the assumptions made earlier in this paper, to an expression for the concentration ahead of the boundary. For the two fastest components the relationship is

$$(V_1-V_2)\,(1+K_2\mathbf{C}_2'+K_1\mathbf{C}_1')\,\mathbf{C}_1' \\ =[V_1\,(1+K_1C_1')-V_2\,(1+K_2\mathbf{C}_2'+K_1\mathbf{C}_1')]\,C_1',$$

where $\mathbf{C}_1', \mathbf{C}_2'$ are the true concentrations behind the boundary of the slower component.

With $1+K_2\mathbf{C}_2'+K_1\mathbf{C}_1'=e$,

$$C_1'=\frac{1}{2K_1}\left[+\left|\sqrt{\left\{\left(1-\frac{V_2}{V_1}e\right)^2+4K_1e\mathbf{C}_1'\left(1-\frac{V^2}{V_1}\right)\right\}}\right| \\ -\left(1-\frac{V_2}{V_1}e\right)\right]. \qquad (5)$$

With $\mathbf{C}_2'/\mathbf{C}_1'=n$, $K_2/K_1=p$, and $V_1/V_2=e$ (i.e. the percentage difference in the mobilities being the same as the percentage increment in the velocity due to the protein concentration behind the boundary), $C_1'=\mathbf{C}_1'\sqrt{(pn+1)}$.

The separation S of the boundaries is given by

$$S=\frac{\mathbf{C}_1'}{C_1'}(V_1-V_2)\,(1+K_1\mathbf{C}_1'+K_2\mathbf{C}_2'). \qquad (6)$$

A limiting case of interest arises when $p=1$ and V_1 nearly equal to V_2; the faster component will adjust itself ahead of the boundary to a concentration nearly equal to the total concentration behind the boundary, independent of the relative concentrations in the mixture. When the mobilities are similar and in addition $p>1$, a gravitationally unstable region may be formed ahead of the boundary of the slower component.

It can be seen by inspection of equation (5) that, by accumulation, the faster component can increase in concentration ahead of a boundary to a multiple of that behind the boundary. It can thus reverse the ratio of the apparent concentrations of two components under conditions normally adhered to, such as, for example, a concentration of proteins of 1 g./100 ml. if the mobilities of the components differ by as much as 5%. The following example illustrates this. Fig. 3 shows the A_1, A_2 (Blix, Tiselius & Svensson, 1941; Hoch & Morris, 1945) and α_2 boundaries after an electrophoresis at pH 8·0, and the αA, βA (Luetscher, 1939), and a small component (possibly α_1) after an experiment at pH 4·0. The apparent concentration of A_1, the fastest component at pH 8·0, is higher than that of A_2. At pH 4·0, where the serum components migrate in the reverse order and where the serum albumin shows two components (αA and βA) having a much greater difference in mobility than that of the two components at pH 8·0, it is also the faster component (αA) which appears higher in concentration. As a consequence of equation (5) these observations suggest that the two components at pH 8·0 were the same as those at pH 4·0 and not that they were

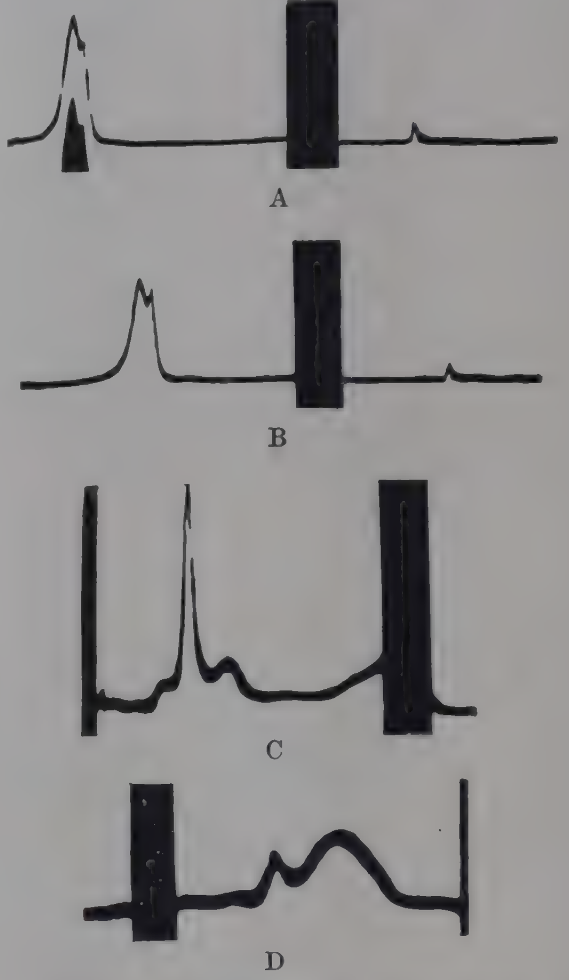

Fig. 3. Apparent proportions of the components of serum albumin. A, B, ascending patterns, showing albumin and α_2 boundaries after prolonged electrophoretic runs on serum. Protein concentration 2·4 g./100 ml.; phosphate buffer pH 8·0, $\mu=0{\cdot}1$. Comparison of B with A shows the reproducibility after the serum had been kept for 16 days at pH 8 in the refrigerator. A, after 550 min. at about 19·5 mA., tilt 67° to the horizontal; B, after 540 min. at 19–19·5 mA., tilt 70°. C ascending and D descending patterns of serum albumin free from α_2, obtained from experiment of Fig. 3B; tilt 25° to the horizontal; protein concentration 0·29 g./100 ml.; acetate buffer pH 4, $\mu=0{\cdot}02$.

different, as might appear from their relative areas, although they may be so for other reasons.

The degree of separation for a particular case of a 2 : 3 mixture of two proteins was calculated for two total concentrations of 1·2 and 0·1 g./100 ml. protein. The velocities at infinite dilution were taken to differ by 7 % ($V_1/V_2=1{\cdot}07$). The degree of separation for the higher concentration (with $K_1=K_2=0{\cdot}06$, $p=1$, and $\mathrm{C}_1'=0{\cdot}48$) works out to 1/1·58 times the separation at infinite dilution. For the lower concentration ($\mathrm{C}_1'=0{\cdot}04$), the degree of separation is very nearly equal to (1/1·04 times) the separation at infinite dilution. If the latter were about 2 mm. one would expect a separation of 1·9 mm. at 0·1 g./100 ml. protein and a separation of 1·27 mm. at 1·2 g./100 ml. protein.

Table 4. *Apparent proportions of the faster component of a two-component mixture, calculated from equations* (5) *and* (7)

	Total concentration (g./100 ml.)						Difference between means
	0·6			2·4			
($V_2/V_1=0{\cdot}4$)	Asc.	Desc.	Mean	Asc.	Desc.	Mean	(%)
	Faster component 0·725 of total						
$K_1=0{\cdot}06$ $K_2=0{\cdot}06$	0·736	0·730	0·733	0·759	0·741	0·750	2·3
$K_1=0{\cdot}06$ $K_2=0{\cdot}03$	0·731	0·730	0·731	0·742	0·741	0·742	1·4
$K_1=0{\cdot}06$ $K_2=0{\cdot}09$	0·742	0·730	0·736	0·776	0·741	0·759	3·0
	Faster component 0·5 of total						
$K_1=0{\cdot}06$ $K_2=0{\cdot}06$	0·516	0·506	0·511	0·545	0·521	0·533	4·2
$K_1=0{\cdot}06$ $K_2=0{\cdot}03$	0·507	0·506	0·507	0·523	0·521	0·522	3·1
$K_1=0{\cdot}06$ $K_2=0{\cdot}09$	0·521	0·506	0·514	0·570	0·521	0·545	5·1

The differences between the values for the total concentrations of 0·6 and 2·4 g./100 ml. are here slightly underestimated since the dependency of K_i on the protein concentration has not been taken into account. The numerical evaluation of equation (4*a*) suggests that a value for K_1 of 0·06 should be increased by about 7 % when the protein concentration rises by 1 g./100 ml.

An example for such conditions might be represented by Fig. 2C. The separation of the two components at the small-albumin boundary is between 2 and 3 mm. At the large-albumin boundary no split could be observed, but a difference in the width of the boundary of about 0·7 mm. was found between patterns B and C (measured at 10–15 mm. above the base line). This corresponds to a spreading of about 1·4 mm. during the experiment between patterns A and C.

In view of the fact that other components with mobilities similar to that of the albumin, apart from A_1 and A_2, may possibly be present in small quantities, the spreading of the large albumin boundary seems in fair agreement with the theory.

The relationship between concentrations of a protein component ahead and behind a descending protein boundary is given by

$$C_2=\frac{1}{2K_2}\left[-\left|\sqrt{\left\{\left(\frac{V_1}{V_2}g-1\right)^2-4K_2g\mathrm{C}_2\left(\frac{V_1}{V_2}-1\right)\right\}}\right|+\left(\frac{V_1}{V_2}g-1\right)\right], \quad (7)$$

where $g=1+K_1\mathrm{C}_1+K_2\mathrm{C}_2$; C_1, C_2 are the true concentrations of the components ahead of the boundary of the faster component.

The extent to which analyses of serum patterns may give values for concentrations that are different from those of the original mixture can be assessed from Table 4, which has been computed from equations (5) and (7) for a two-component system simulating one containing an albumin and a globulin component, of mobilities in the ratio 1 : 0·4. The calculation was carried out for different values of K_2 and for two relative proportions of the two components, one being similar to that given in Table 2 for $(A+\alpha_1+\alpha_2):(\beta+\gamma)$, i.e. the faster component 72·5 % of the total. The concentrations on the ascending side were calculated from those assumed for the original mixture by multiplication by ρ. Although ρ is dependent on K_2, K_1 being kept constant, a change in ρ of 5 or 10 % affects the values given in Table 4 only slightly, and therefore ρ was kept the same for all values of K_2. The values used,

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0·97 and 0·87 for the lower and higher concentrations respectively, were average experimental values found for serum.

SUMMARY

1. The anomalies in electrophoretic patterns of protein mixtures are considered theoretically for specified conditions, and the equations derived are applied to the study of normal and pathological sera.

2. The value for a coefficient which relates the change in migration velocity of protein ions to the change in protein concentration was determined experimentally for human serum albumin. It was found to be $K_1 = 0{\cdot}06$, signifying an increase of 6 % in the migration velocity of an ascending boundary/g. protein/100 ml.

3. The average net charge of the albumin was calculated by inserting this value into an expression derived from the equation for the conductivity change across a colloid boundary given by Svensson (1946). A value for the valence of about -19 was found, which is consistent with that of -18, quoted by Cohn *et al.* (1944) for pH 7·4, obtained from measurements of electromotive force.

4. The α-globulin boundary in patterns of serum in phosphate buffer at pH 8·0 and $\mu = 0{\cdot}1$ was found to have a slightly anomalous position relative to that of the albumin boundary.

5. A method for comparing mobilities by simultaneous measurements is outlined.

6. The calculation of the concentration relationships in a boundary region of two components reveals that the relative concentrations as deduced from the apparent concentration changes at the boundaries give a grossly erroneous picture of the concentrations in the original solution, when the value for the relative difference in the mobilities is in the region of that of K_1 or lower.

7. The degree of separation of two boundaries depends on the total concentration. The examination for electrophoretic homogeneity should be carried out at low concentrations where the separation of the boundaries is greatest and over a range of concentrations as far as the interference of diffusion allows. An experiment on human serum is described in which separation of the albumin boundary into two components was observed at a concentration of 0·07 % after a migration of 31 mm., while no separation but only broadening was seen at a concentration of 1·2 %.

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While this paper was being prepared for press, a publication by Longsworth (1947) appeared dealing with related problems.

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The Use of Phenol Glucuronide in the Assay of β-Glucuronidase

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Our present knowledge of the distribution in the body and the physical properties of β-glucuronidase is derived almost entirely from experiments, in which free glucuronic acid was determined by reduction of ferricyanide after enzymic hydrolysis of a conjugated glucuronide. Whilst it is possible to determine very small amounts of glucuronic acid by means of its reducing power (Levvy, 1946), the lack of specificity in the reaction is a serious disadvantage in the routine assay of glucuronidase. Unless the enzyme preparation has been considerably purified, it may contain large amounts of materials other than glucuronic acid which reduce ferricyanide (Levvy, 1948). Other impurities may interfere during the actual titration, if this is cerimetric, obscuring the end point and making it unstable. In studying the enzymic hydrolysis of oestriol glucuronide, Fishman (1939) determined free oestriol by the Kober colour reaction. Its scarcity, however, renders this substrate unsuitable for routine assays. Talalay, Fishman & Huggins (1946) have recently described a method whereby the hydrolysis of phenolphthalein glucuronide can be followed colorimetrically. This method appears to avoid the difficulties mentioned above in the determination of glucuronidase activities. It was found possible in this laboratory to apply the method of King & Armstrong (1934) for phosphatase determination to the assay of glucuronidase, using biosynthetic phenol glucuronide (Williams, private communication) as substrate. (We are greatly indebted to Dr Williams for details of the technique for obtaining this compound from rabbit urine.) Results obtained in studying the kinetics of hydrolysis of phenol glucuronide with enzyme obtained from two different organs, and a description of the procedure for the routine assay of glucuronidase in mouse tissues, are given below.

EXPERIMENTAL

Preparation of phenol β-D-glucuronide. The glucuronide was obtained from the urine of rabbits fed with phenol by the general procedure described by Williams (1943) for the isolation of aminophenol glucuronides. Before use, it was dried over P_2O_5 *in vacuo* for 10 hr. at 80°. Analysis showed it to be free from solvent of crystallization (m.p. 162°, corr.).

The colour reaction for free phenol. Phenol was determined with the reagent of Folin & Ciocalteu (1927). King & Armstrong (1934) give full details of the technique for this estimation. The colour intensity was measured with a Spekker photoelectric absorptiometer, using Ilford no. 602 blue filter. Free glucuronic acid did not interfere in the reaction. Phenol glucuronide in high concentration gave a faint colour, probably due to traces of free phenol (less than 0·2% of the total weight), since it varied from one sample to another. Contrary to the findings of Folley & Kay (1935), the reaction was not particularly sensitive to the Na_2CO_3 concentration and was complete in less than 20 min. at 37°. In constructing the calibration curve (Fig. 1), 0·6 ml. standard phenol solution (Hawk & Bergeim, 1931) was added to 0·2 ml. 0·1 M-citrate buffer (pH 5·2), followed by 2 ml. of a 1 : 5 dilution of the Folin-Ciocalteu reagent. After mixing, 2 ml. of the liquid were taken for colour development as described below for enzyme extracts. Citrate, acetate and formate buffers had no effect on the colour reaction.

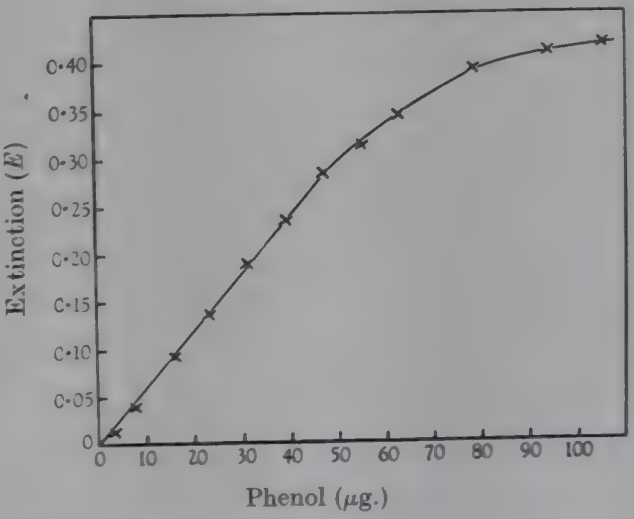

Fig. 1. Calibration curve for estimation of phenol.

Preparation of the enzyme. Preparations from mouse liver and spleen were studied separately in these experiments. The organs were broken up in glass homogenizers (Umbreit, Burris & Stauffer, 1945). Unless stated to the contrary, the enzyme was partially purified following the method described by Talalay *et al.* (1946). The homogenate was brought to pH 5·0 or 5·2 with acetic or citric acid. Protein which separated after 30 min. at 37° was removed by centrifuging, and the enzyme in the supernatant liquid precipitated by adding an equal volume of saturated

$(NH_4)_2SO_4$ solution (SAS). The precipitate was dissolved in a convenient volume of water. No change in the amount of enzyme present was noted after purification in this way.

The effect of pH. The pH for optimum hydrolysis was determined over the range 3–6·5 at a substrate concentration of 0·01 M in citrate buffer. To 0·2 ml. of 0·1 M-buffer and 0·2 ml. of 0·04 M-substrate warmed to 37° was added 0·4 ml. enzyme, and the mixture was incubated at 37° for 1 hr. The initial effect of substrate and enzyme on the pH of the buffer was determined electrometrically. No further change in the pH occurred during the incubation.

With crude homogenates of liver and spleen, considerable amounts of substances giving the colour reaction were liberated in absence of substrate. It would appear that an enzymic process was responsible for the production of these compounds since the amount formed varied with the pH, with an optimum at 3·6–4·6. Partial purification of the homogenates as described above made the blank much smaller and very nearly independent of the pH. With purified enzyme from liver and spleen, the curves for variation in hydrolysis with pH assumed the shapes shown in Fig. 2. Homogenates from five mice were pooled for each organ before purifying, and all points on the curve were determined simultaneously. Corrections were applied for the enzyme blank at the appropriate pH (corresponding to about 200 μg. phenol/g. for spleen and about 100 μg./g. for liver) before plotting the points. From Fig. 2 it can be seen that for enzyme from both sources the rate of hydrolysis rose to a maximum at pH 5·2 as the pH was lowered. After a drop at pH 4·75, the velocity again rose slightly at pH 4·5 and then fell off rapidly. The subsidiary peak at pH 4·5 was seen in other experiments when the pH increment was made small enough. A possible explanation of the shape of the curves in Fig. 2 is given by the work of Mills (1947), who found that β-glucuronidase from beef spleen could be separated into two fractions with slightly different pH optima for the hydrolysis of menthol glucuronide. In acetate buffer, some results were obtained which suggested the pH optimum to be lower than in citrate, but this point was not further investigated. It was decided that for the routine assay of the enzyme the hydrolysis of phenol glucuronide should be done at pH 5·2 in citrate buffer.

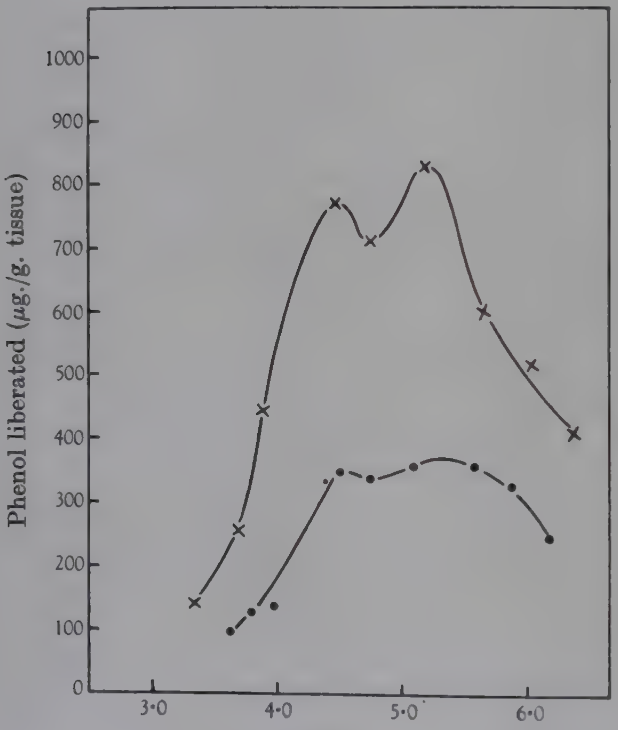

Fig. 2. The variation in hydrolysis rate of phenol β-D-glucuronide with pH. Results expressed in terms of phenol liberated/g. moist tissue. ×—× spleen enzyme; ●—● liver enzyme.

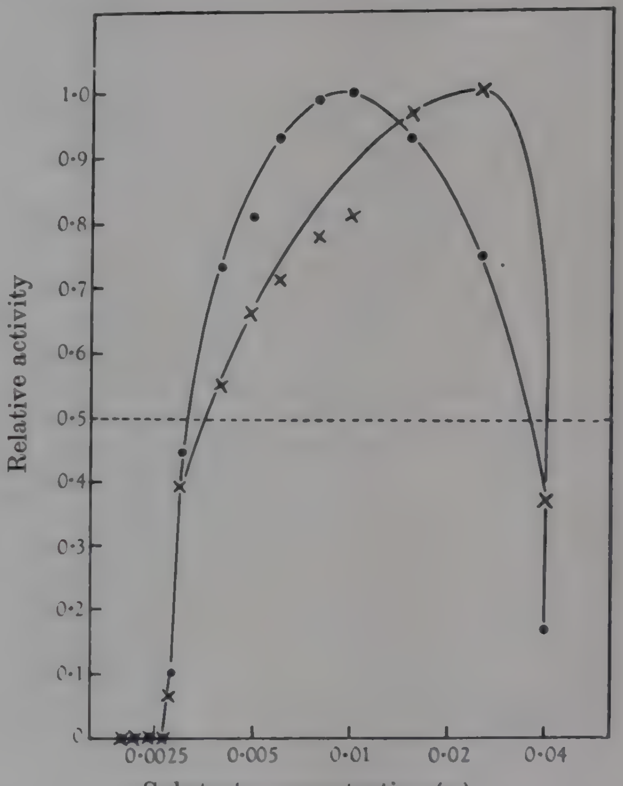

Fig. 3. The variation in initial hydrolysis rate with substrate concentration. Results expressed as fraction of maximum observed activity. ×—× spleen enzyme; ●—● liver enzyme.

The effect of substrate concentration. The effect of varying the substrate concentration at constant pH is shown in Fig. 3. The citrate buffer and substrate solutions were adjusted to pH 5·2. Addition of enzyme caused no appreciable change in pH. Three experiments were done with enzyme from each source and the averaged results are shown in the figure. It will be noted that inhibition by excess substrate was marked in both cases. The substrate optimum for spleen (0·025M) was greater than for liver (0·01M). The results did not lend themselves to the accurate calculation of the Michaelis-Menten constant, K_m, by the method of Lineweaver & Burk (1934), but the value obtained graphically from Fig. 3 by finding the substrate concentration at which half the observed maximum velocity of hydrolysis was developed was approximately the same (0·0035M) for enzyme from either source. The difference in shape between the curves for liver and spleen may be due to the presence of interfering substances in the tissue extracts rather than a qualitative difference in the enzyme system. A substrate concentration of 0·015M was adopted for further work with enzyme from both sources.

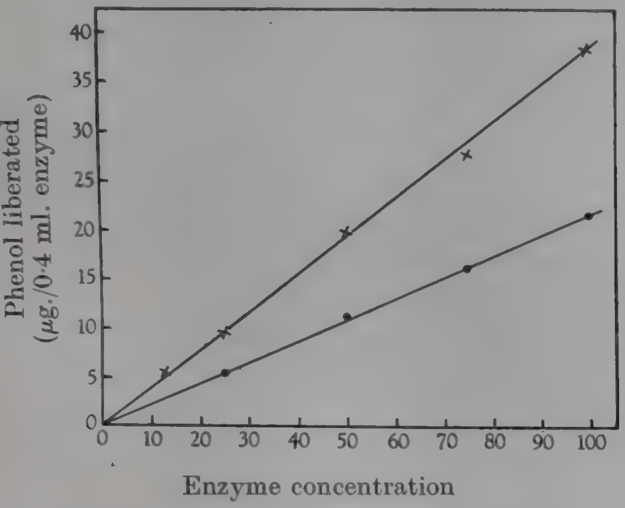

Fig. 4. The relation between enzyme concentration and phenol liberated under standard conditions. Enzyme concentration in arbitrary units. ×—× spleen enzyme; ●—● liver enzyme.

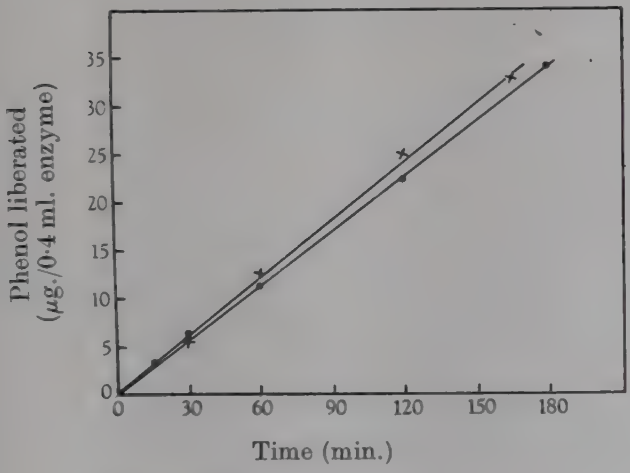

Fig. 5. Phenol liberated after varying incubation periods. ×—× spleen enzyme; ●—● liver enzyme.

The effect of varying the enzyme concentration and the duration of hydrolysis. As shown in Fig. 4, the amount of phenol liberated in 1 hr. at 37° and pH 5·2 (citrate buffer) from 0·015M-phenol glucuronide was directly proportional to the enzyme concentration. Experiments carried out under the same conditions, but in which the incubation period was varied and the enzyme concentration kept constant, showed the reaction velocity to be unchanged after 3 hr. (Fig. 5).

The assay of β-glucuronidase

Reagents

Citrate buffer. (*a*) 0·3M, pH 5·2 (glass electrode); (*b*) 0·1M, prepared from 0·3M.

Substrate solution. 0·06M-Phenol glucuronide adjusted to pH 5·2; stored in the refrigerator.

Folin-Ciocalteu reagent. (*a*) Stock solution (British Drug Houses Ltd.); (*b*) Stock solution diluted 1 to 5 and prepared daily.

N-*Sodium carbonate solution.*

Saturated ammonium sulphate solution. Adjusted to pH 5·2.

Procedure

Spleen. The dissected organ was weighed in a chilled homogenizer tube and homogenized with 3 ml. water. The cell-free homogenate was transferred to a graduated centrifuge tube, using 1 ml. wash water. After addition of 0·5 ml. 0·3M-buffer, the tube was maintained at 37° for 30 min. Coagulated protein was separated by centrifuging for 15 min. The volumes of precipitate and supernatant liquid were noted and the latter was transferred to another graduated centrifuge tube. An equal volume of SAS was added and the tube centrifuged for 30 min. The liquid was discarded, and the residue dissolved in water and made up to a volume of 2 ml. The concentration of enzyme was then such that, in the case of normal animals, 0·4 ml. of the solution gave readings of 20–40 μg. phenol after corrections had been applied for enzyme and substrate blanks. This volume of the enzyme solution was added to 0·2 ml. of 0·1M-buffer and 0·2 ml. substrate in a stoppered 10 ml. centrifuge tube. Prior to addition of the enzyme, the substrate and buffer were warmed to 37°. After incubation of the hydrolysis mixture for 1 hr. at 37°, 2 ml. of the diluted Folin-Ciocalteu reagent were measured into the tube. Protein was removed by centrifuging for 3 min. and 2 ml. of the supernatant transferred to a 10 ml. stoppered centrifuge tube containing 4 ml. Na_2CO_3 solution. The contents of the tube were mixed and the colour developed by incubating for 20 min. at 37°. Assays were carried out in duplicate.

Liver. The procedure was exactly as described above except that at certain stages the volumes used differed. Homogenization was done with 5 ml. water and 3 ml. of water were added in transferring the homogenate to the first graduated tube. In adjusting the pH, 1 ml. 0·3M-buffer was used. The solution of the enzyme after SAS precipitation was made up to 4 ml., this volume giving readings of 20–40 μg. phenol in the assay after correction for blanks with livers from normal animals.

Controls. Every assay was controlled by incubating mixtures of enzyme and buffer containing 0·2 ml. water in place of substrate. The purified enzyme shows a small rise in the blank during incubation. Controls for phenol in the substrate were also done at frequent intervals, 0·2 ml. of the solution being incubated with 0·2 ml. buffer and 0·4 ml. water. Since there appeared to be a very small but perceptible increase in the reading when the substrate was incubated at pH 5·2, this procedure was considered preferable to that in which substrate is added to the enzyme after incubation (cf. Talalay *et al.* 1946).

RESULTS

The recoveries obtained in a series of experiments in which 0·2 ml. standard phenol solution was added to 0·2 ml. 0·1 M-buffer and 0·4 ml. enzyme (spleen and liver mixed) are shown in Table 1. In some experiments the mixture was incubated whilst in others the Folin-Ciocalteu reagent was added at once. Each figure shown in the table is the result of a determination carried out in duplicate and the results have been corrected for the enzyme blank (5·2 μg. unincubated, 7·7 μg. incubated).

Table 1. *Recovery of phenol added to buffered enzyme*

Phenol added	Phenol recovered before incubation		Phenol recovered after incubation	
(μg.)	(μg.)	(%)	(μg.)	(%)
3·9	3·7	95	4·1	105
7·9	8·0	101	8·0	101
15·7	15·6	99	15·8	101
31·4	31·1	99	31·2	99
62·9	63·1	100	63·0	100
105·0	105·0	100	104·9	100

In a series of 30 determinations in which 31·4 μg. phenol were added to enzyme and buffer as above, and determined at once, the mean recovery was 31·5 μg. (100·3 %), and the standard deviation of a single determination from the mean was 0·19 μg. (0·6 %).

DISCUSSION

From the results given above it can be seen that phenol glucuronide is a convenient substrate for the assay of β-glucuronidase, using the reagent of Folin & Ciocalteu for estimation of phenol liberated by the enzyme. As compared with phenolphthalein glucuronide (Talalay *et al.* 1946), a much higher concentration of phenol glucuronide is required for optimum hydrolysis, and inhibition of the enzyme by excess substrate is more pronounced.

It has been generally assumed without any experimental justification that the conditions for optimum hydrolysis by β-glucuronidase are always the same no matter the source of the enzyme. The data obtained in this work for mouse liver and spleen glucuronidase support the assumption. Masamune (1934), in his pioneer work on glucuronidase, found hydrolysis of phenol glucuronide by beef kidney extracts, determined by estimation of glucuronic acid, to be at a maximum at pH 5·3–5·6 in citrate buffer. This is in good agreement with our own figure of 5·2 for mouse liver and spleen extracts.

SUMMARY

1. Phenol glucuronide is a convenient substrate for the assay of β-glucuronidase, using the Folin & Ciocalteu (1927) reagent for the estimation of free phenol.

2. In the procedure adopted for assay of glucuronidase in mouse liver and spleen, hydrolysis of phenol glucuronide was carried out in citrate buffer at pH 5·2 and a substrate concentration of 0·015 M.

3. The mean recovery of added phenol in the assay procedure was 100 %, and the standard deviation of a single observation from the mean was 0·6 %.

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Studies in Vitamin A

4. SPECTROPHOTOMETRIC DETERMINATION OF VITAMIN A IN LIVER OILS. CORRECTION FOR IRRELEVANT ABSORPTION

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The ultraviolet absorption spectrum of vitamin A with its maximum near 328 mμ. was described nearly 20 years ago (Morton & Heilbron, 1928*a*, *b*). The intensity of absorption shown at that wave length by a fish-liver oil or concentrate was conveniently expressed in terms of $E^{1\%}_{1\,\text{cm.}}$ 328 mμ. (Gillam & Morton, 1929), and this magnitude was early seen to afford a guide to potency. If a number of fish-liver oils covering a fair range of potency was examined in three different ways, namely, (*a*) by the method of biological assay, (*b*) by the antimony trichloride colour test, and (*c*) by the spectrophotometric method, the results showed fair agreement. From a strictly quantitative standpoint the bio-assays were primitive, the colour test was handicapped by a highly variable degree of inhibition and the ultraviolet absorption by superimposed irrelevant absorption.

Experience gained by a very large number of workers has since shown that bio-assays cannot compete with the spectrophotometric method in terms of either convenience or accuracy. In fact, for a bio-assay to be worth doing to-day a very large number of animals needs to be used, and the data must be subjected to rigorous statistical study. This makes it all the more necessary to dispose of difficulties and complications in the spectroscopic procedure.

The broader aspects of the problem of vitamin A activity raise acute difficulties (Morton, 1942; Hickman, 1943; Gridgeman, 1944), and it is proposed at this stage to confine attention mainly to the optimal use of spectrophotometry. We now enjoy advantages which were previously lacking. Vitamin A and its acetate are now available as pure, crystalline substances, and photoelectric spectrophotometry is capable of a higher degree of accuracy than photographic methods. Irrelevant absorption can be allowed for (Morton & Stubbs, 1946) so that the true intensity of absorption attributable to the vitamin A can be determined. By the use of such a correction procedure one major difficulty has been removed. This difficulty concerned the relationship between $E^{1\%}_{1\,\text{cm.}}$ 328 mμ. and potency determined by large-scale co-operative feeding trials with (*a*) halibut-liver oil and (*b*) crystalline vitamin A β-naphthoate. The conversion factors differed significantly, but the discrepancy disappeared when the gross intensity of absorption shown by the halibut-liver oil was suitably corrected for irrelevant absorption (Morton & Stubbs, 1947).

The purely analytical problem of determining the vitamin A content of an oil does not, however, of necessity require any reference to conversion factors or to the international unit of vitamin A activity (0·6 μg. of β-carotene). From many points of view further studies on the wider problem would be made easier if 'potencies' could be replaced by 'vitamin content' expressed in mg./g.

Spectrophotometric characteristics of vitamin A (free alcohol) and vitamin A acetate

Any attempt to determine vitamin A in an oil (where it normally occurs mainly as esters), or in an unsaponifiable fraction, requires at least accurate values for the molecular extinction coefficient (ϵ) or $E^{1\%}_{1\,\text{cm.}}$ at the maximum for the free or esterified vitamin dissolved in a specified solvent. For the extended use of spectrophotometry the whole absorption curve needs to be known somewhat accurately so that irrelevant absorption can be estimated.

Crystalline vitamin A acetate dissolved in *cyclo*hexane shows a maximum at 328 mμ. The intensities of absorption at different wave lengths are shown in Table 1 and are expressed as fractions of the peak value. The two wave lengths, one on each side of the maximum, at which the intensity of absorption is exactly 6/7 of the peak value have been determined as carefully as possible as 313 and 338·5 mμ. The same wave lengths apply to the purest natural ester preparations (Morton & Stubbs, 1946). It is to be noted that these figures apply to *cyclo*hexane solutions, because the absorption spectra of vitamin A and its esters vary a little from solvent to solvent. The absorption curve for the free vitamin differs a little from that of the esterified vitamin. Small though the difference is, it must be taken into account in applying correction procedures. Table 1 contains enough information for most purposes. The only significance of the ratio 6/7 is that it is empirically appropriate in relation to the wave length range covered and to the performance of the spectrophotometer.

Table 1. *Intensities of absorption of vitamin A and vitamin A acetate in different solvents, expressed as a fraction of* $E_{\text{max.}}$ *for wave lengths between 220 and 450* mμ.

λ (mμ.)	Crystalline vitamin A alcohol		Crystalline vitamin A acetate		
	*cyclo*Hexane	Ethanol	*cyclo*Hexane	Ethanol	75% (v/v) aqueous ethanol
220	0·143	0·089	—	0·106	—
230	0·127	0·093	—	0·106	—
240	0·128	0·102	—	0·113	—
245	—	0·107	—	—	—
250	0·125	0·112	0·135	0·123	0·123
255	0·126	0·112	0·137	—	—
260	0·123	0·109	0·136	0·122	0·133
265	0·127	0·115	0·138	—	—
270	0·150	0·139	0·158	0·150	—
275	0·187	0·175	0·193	—	—
280	0·236	0·224	0·238	0·254	0·265
285	0·300	0·313	0·300	0·337	—
290	0·393	0·414	0·373	0·410	0·405
295	0·515	0·518	0·469	0·514	—
300	0·611	0·609	0·571	0·604	0·592
305	0·718	0·720	0·668	0·716	—
310	0·841	0·844	0·795	0·826	0·814
311	0·861	0·857	—	0·845	—
311·5	—	—	—	0·854	0·840
312	0·877	—	0·838	0·864	0·844
312·5	—	—	—	—	0·858
313	0·888	—	0·858	0·878	0·863
315	0·906	0·898	0·889	0·897	0·888
317·5	0·923	0·922	0·915	0·927	—
320	0·950	0·950	0·934	0·953	0·946
322·5	0·978	0·983	0·961	—	—
325	0·998	1·000	0·988	0·999	0·991
326	1·000	1·000	—	1·000	0·999
326·5	—	—	—	—	1·000
327	0·998	0·990	—	1·000	0·999
328	0·994	—	1·000	0·990	0·991
330	0·972	0·957	0·995	0·976	0·982
335	0·880	0·869	0·925	0·905	0·914
335·5	—	0·861	—	—	—
336	0·861	0·849	—	0·886	—
337	—	—	—	0·868	—
337·5	—	—	—	0·860	0·872
338	—	—	—	0·852	—
338·5	0·810	—	0·857	—	0·855
340	0·776	0·772	0·828	0·812	0·827
345	0·646	0·638	0·707	0·689	—
350	0·513	0·509	0·572	0·557	0·580
355	—	0·397	0·440	0·437	—
360	0·293	0·278	0·331	0·317	0·359
365	0·207	0·172	0·239	—	—
370	0·129	0·108	0·151	0·132	0·175
375	—	0·074	—	—	—
380	0·046	0·048	—	0·043	—
390	0·025	0·024	—	0·013	—
400	0·012	0·013	—	0·004	—
450	0·0003	0·0018	—	0·0002	—
	$\lambda_{\text{max.}}$ 326 mμ.	$\lambda_{\text{max.}}$ 325 mμ.	$\lambda_{\text{max.}}$ 328 mμ.	$\lambda_{\text{max.}}$ 326·5 mμ.	$\lambda_{\text{max.}}$ 326·5 mμ.
	$\epsilon_{\text{max.}}$ 48,310	$\epsilon_{\text{max.}}$ 51,100	$\epsilon_{\text{max.}}$ 48,460	$\epsilon_{\text{max.}}$ 50,020	$\epsilon_{\text{max.}}$ 50,510
	$E_{311}=E_{336}$	$E_{311}=E_{335\cdot5}$	$E_{313}=E_{338\cdot5}$	$E_{311\cdot5}=E_{337\cdot5}$	$E_{312\cdot5}=E_{338\cdot5}$
	$\frac{E_{326}}{E_{311}}=7/6$	$\frac{E_{325}}{E_{311}}=7/6$	$\frac{E_{328}}{E_{313}}=7/6$	$\frac{E_{326\cdot5}}{E_{311\cdot5}}=7/6$	$\frac{E_{326\cdot5}}{E_{312\cdot5}}=7/6$

The need for correction of data on liver oils

For many years it has been the custom to prepare the unsaponifiable fractions of low-potency oils prior to making spectrophotometric readings. It is not necessary to weigh the unsaponifiable fraction, as the intensity of absorption is more conveniently expressed in terms of the $E^{1\%}_{1\,\text{cm.}}$ value calculated on the weight of oil used. As compared with readings made on unsaponified oils, $E_{\text{max.}}$ is significantly reduced in low-potency oils, and the whole absorption curve approximates in shape to that shown by high-potency oils and rich concentrates. It was not unreasonable to assume that substances giving rise to irrelevant absorption and rendered water soluble by saponification would occur to roughly the same extent in all fish-liver oils. If that were the case such irrelevant absorption would constitute a negligible fraction of the total absorption at 328 mμ. in rich oils. Support for the assumption was provided by the observation that practically all the absorbing material of rich oils remained in the unsaponifiable fraction.

The advantage of the data in Table 1 is that the presence of irrelevant absorption becomes easy to detect. If the sample under test, e.g. a fish-liver oil, is free from irrelevant absorption the line joining the 313 and 338·5 mμ. points on its absorption curve will be horizontal at a level 6/7 of the intensity at 328 mμ. (esterified vitamin A in *cyclo*hexane). This condition is rarely satisfied, and some correction is needed even with high-potency oils. A good example is provided by the halibut-liver oils assayed carefully in 1936 and retested spectrophotometrically in 1946 (Morton & Stubbs, 1947).

The reason why irrelevant absorption occurs so frequently in high-potency oils is that vitamin A decomposition products are formed either before or during the process of extracting livers. There may also be deterioration during transit or storage. Whatever the exact reason for their occurrence, the fact of contamination by such artifacts is undeniable. Most of the decomposition products remain in the unsaponifiable fraction.

Correction procedure

The simple correction procedure described by Morton & Stubbs (1946) rests upon the assumption that the irrelevant absorption 'curve' is linear over the approximate range 310–340 mμ., i.e. that no impurity or artefact shows a maximum very close to that of vitamin A. A good deal is now known concerning the spectra of vitamin A_2 and oxidation products of vitamin A, and it can be said that over the range 310–340 mμ. their absorption curves are at least approximately linear. Nevertheless, slight departures from linearity could give rise to appreciable errors in the final result, and the final verdict on the validity of the assumption must rest on the kind of experience recorded below.

The three chosen wave lengths, $\lambda_{\text{max.}}$ for vitamin A alcohol or ester in a specified solvent and the two at which the E value is 6/7 of $E_{\text{max.}}$, may be called for convenience 'fixation points'. With a known concentration of oil or concentrate (in the specified solvent), such that $E'_{\text{max.}}$ for a 1 cm. cell occurs in the region of optimal performance for the photoelectric spectrophotometer, E values are determined at the three fixation points. Normally the values on either side of the maximum are different and both exceed 6/7 of the peak value. The correction is made in two steps as in the example given below:

Cod-liver oil, 1 % solution in *cyclo*hexane:

mμ.	E	Δ mμ.	
313	0·640	15	25·5
328	0·712	10·5	
338·5	0·620		

(i) $E_{313\,\text{m}\mu.} - E_{338\cdot5\,\text{m}\mu.} = 0\cdot020$:

By similar triangles the correction for slope is

$$\frac{0\cdot020 \times 10\cdot5}{25\cdot5} = 0\cdot008,$$

$$0\cdot712 - 0\cdot008 = 0\cdot704.$$

(ii) Correction for the height of the general absorption:

$$\frac{0\cdot704 - x}{0\cdot620 - x} = \frac{7}{6}, \quad x = 0\cdot116,$$

$$0\cdot704 - 0\cdot116 = 0\cdot588,$$

$$E^{1\%}_{1\,\text{cm.}}\ 328\ \text{m}\mu.\ (0\cdot712\ \text{gross}) = 0\cdot588\ (\text{corrected}).$$

Given this one point, i.e. E 328 mμ., corrected, the whole corrected vitamin A absorption curve can be constructed from the data in Table 1. The difference between this curve and the uncorrected curve will represent the spectrum of substances other than vitamin A, provided that the assumption of linearity over the fixation range is valid.

It will be convenient first to consider tests carried out on liver oils from the tunny (*Thunnus thynnus*). Portuguese workers (Gouveia, Coelho, Gouveia & Paz, 1943), in confirmation of other experience, have recorded high figures for tunny-liver oils. In some fishes, livers weighing more than 1 kg. yielded 10 % of oil, some 40 % of which seemed to consist of vitamin A esters. The absorption curves of commercial tunny-liver oils now being imported into this country appear at first sight 'normal' enough to need little correction, but when judged by the criteria of Table 1 substantial correction is seen to be essential. Granted that the uncorrected curve fails to satisfy the fixation criteria it is desirable to provide independent checks on the correction procedure, and so indirectly to test the validity of the assumption that irrelevant absorption is linear over the fixation range.

If the correction procedure is found to withstand successfully the most searching tests which can be devised, it will then be of interest to ascertain the magnitude of the correction appropriate to some of the principal types of fish-liver oils and concentrates.

The next step will be to subject the correction procedure to the severe test of indicating correctly the proportion of vitamin A_2 in liver oils which contain both vitamin A and vitamin A_2.

The most severe test of all will be to apply the procedure to whale-liver oils, first those of high quality, i.e. refined oils in which the main absorbing constituent other than vitamin A is kitol, and secondly to crude oils in which the gross absorption is several times greater than that properly attributable to vitamin A. If the method can bear this weight of interpretation the purely analytical problem will have been solved and the way cleared for studies concerned with utilization of vitamin A in the body.

RESULTS

The Beckman ultraviolet photoelectric spectrophotometer has been used throughout.

Spectroscopic data on high-potency tunny-liver oils

Sample A

(i) Solution in *cyclo*hexane, 0·001%; $\lambda_{\max.}$ occurred at 328 mμ., $E^{1\%}_{1\,\text{cm.}}$ 328 mμ., 482 (gross); inflexions near 370 and 392 mμ., indicative of 'cyclized' or anhydro-vitamin A, were observed. $E^{1\%}_{1\,\text{cm.}}$ 328 mμ., 400 (corrected on the basis of esterified vitamin A). The subtraction curve was almost horizontal at 310–340 mμ., but irrelevant absorption was considerable, because of the presence both of 'cyclized' vitamin A and oxidation products (Fig. 1).

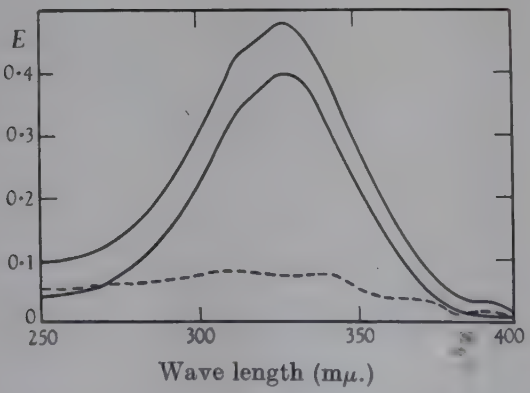

Fig. 1. Absorption curve for high-potency tunny-liver oil; upper curve observed on 0·001% solution in *cyclo*hexane; lower curve corrected vitamin A absorption; broken curve, irrelevant absorption. 1 cm. cell.

(ii) A portion of oil (0·5 g.) was saponified; yield of unsaponifiable fraction 56%, $E^{1\%}_{1\,\text{cm.}}$ 326 mμ., 802; $SbCl_3$ colour test: $E^{1\%}_{1\,\text{cm.}}$ 693 mμ., 168; 618 mμ., 2300; 582 mμ., 1105; some vitamin A_2 was present because the 693 mμ. maximum was seen clearly. $E^{1\%}_{1\,\text{cm.}}$ 326 mμ. (corrected on basis of free vitamin A) 637; recalculated on weight of oil taken, 357.

(iii) A portion of oil (0·5 g.) was saponified in the presence of a little quinol as anti-oxidant; yield of unsaponifiable matter 57·2%; $\lambda_{\max.}$ occurred at 326 mμ. (*cyclo*hexane); $E^{1\%}_{1\,\text{cm.}}$ 326 mμ., 791, or 452·5 calculated on oil; $SbCl_3$ test $E^{1\%}_{1\,\text{cm.}}$ 693 mμ., 160; 618 mμ., 2226; 582 mμ., 1094. $E^{1\%}_{1\,\text{cm.}}$ 326 mμ., 661 (corrected), or 378 calculated on oil (Fig. 2).

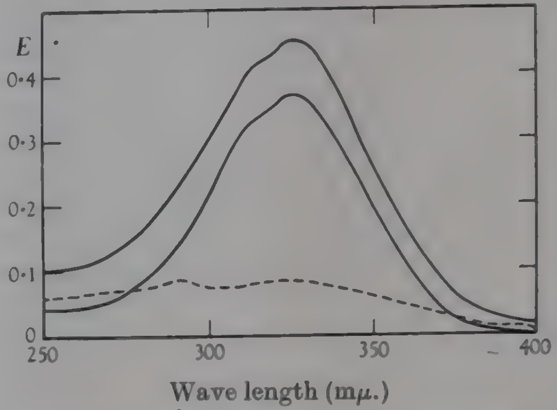

Fig. 2. Absorption curve for unsaponifiable fraction of the tunny-liver oil of Fig. 1, 0·001%, calculated on weight of oil used; upper curve observed, lower curve corrected vitamin A absorption; broken curve, irrelevant absorption. 1 cm. cell.

(iv) Unsaponifiable matter (0·2655 g.) was partitioned between 83% (v/v) aqueous ethanol and light petroleum: (*a*) ethanolic fraction, $E^{1\%}_{1\,\text{cm.}}$ 326 mμ., 599 (gross), 434 (corrected), calculated on fraction (0·2086 g.); (*b*) light petroleum fraction (0·057 g.), $E^{1\%}_{1\,\text{cm.}}$ 295 mμ., 47; 350 mμ., 22·2; 370 mμ. 16·5; 390 mμ., 8·5. The E values are here expressed on the weight of crude unsaponifiable matter; the absorption curve is uncorrectable. The fraction (*b*) seemed to be rich in 'cyclized' vitamin A but free from the vitamin itself. Appreciable losses of vitamin A occurred in this experiment.

(v) After standing for some weeks the small remaining amount of sample A had deteriorated appreciably: $E^{1\%}_{1\,\text{cm.}}$ 328 mμ., 403 (gross), 318 (corrected). A portion, 0·1 g., was saponified and the unsaponifiable fraction extracted with 250 ml. of 50% (v/v) aqueous ethanol by warming on a water bath with frequent shaking. The extract was filtered through kieselguhr, and the combined extract and washings made up to 500 ml. with absolute ethanol. $E^{1\%}_{1\,\text{cm.}}$ 325 mμ., 369 (gross); 315 (corrected). The 50% ethanol thus dissolves all the vitamin A but only a small part of the material contributing irrelevant absorption.

Sample B

(i) Solution in *cyclo*hexane (0·001%), $E^{1\%}_{1\,\text{cm.}}$ 328 mμ., 484 (gross); 397 corrected on the basis of esterified vitamin A as the predominant form.

(ii) A portion of oil (0·05 g.) in 10 ml. peroxide-free ether was poured on to a column of silver sand (20 × 1·5 cm.). The solution dispersed the oil at the top quarter of the column and the solvent was removed by means of an upward current of nitrogen. The column was then washed twice with 90 ml. portions of 70% (v/v) ethanol-water. (*a*) The first washing was diluted 1 in 5 with absolute ethanol, after first making up to 100 ml. ($c=0{\cdot}001\%$ calculated on the weight of oil used initially.) $E^{1\%}_{\text{cm.}}$ 326·5 mμ., 49·3 (gross), 34·3 (corrected). Maxima at 370 and 392 mμ. due to anhydro-vitamin A were shown clearly. (*b*) The second washing, made up to 100 ml. was diluted 1 in 5 (absolute ethanol). $E^{1\%}_{1\text{ cm.}}$ 326·5 mμ., 13·8 (gross), 9·8 (corrected). Bands at 370 and 392 mμ., were again shown. The column was then washed with five successive portions of 80% (v/v) ethanol-water. Each was made up to 100 ml. and appropriately diluted for spectrophotometric tests. The following results all refer to concentrations expressed in terms of the initial weight of oil:

	$E^{1\%}_{1\text{ cm.}}$ 326·5 mμ.	
	Gross	Corrected
(*c*)	54·2	49·0
(*d*)	51·3	45·5
(*e*)	34·5	31·5
(*f*)	28·6	25·2
(*g*)	20·5	17·5

The column was then washed with absolute ethanol (90 ml.) and the washings made up to 200 ml. A portion was diluted twentyfold. $E^{1\%}_{1\text{ cm.}}$ 326·5 mμ., 200·8 (gross), 156·8 (corrected). By adding together the E values for all the fractions, the total recovery was $E^{1\%}_{1\text{ cm.}}$ 326·5 mμ., 453 (gross), 370 (corrected). A repetition of the experiment gave a total recovery of $E^{1\%}_{1\text{ cm.}}$ 326·5 mμ., 451 (gross).

(iii) A quantity of oil (0·1323 g.) was saponified. The unsaponifiable fraction was extracted with 250 ml. of 50% (v/v) aqueous ethanol by warming on a water bath with frequent shaking for 0·5 hr. The solution was filtered through kieselguhr and the latter was washed with 100 ml. of 50% ethanol. The combined filtrate and washings were made up to 500 ml. with absolute ethanol. The material retained by the kieselguhr was washed by means of redistilled ether into the flask containing the matter insoluble in 50% ethanol. The solvent was removed and the residue dissolved in *cyclo*hexane:

(*a*) Fraction soluble in 50% ethanol. $E^{1\%}_{1\text{ cm.}}$ 325 mμ., 427 (gross), 392 corrected (Fig. 3).

(*b*) Remainder of unsaponifiable matter, $\lambda_{\text{max.}}$ 305 mμ., inflexions at 370 and 392 mμ. indicating the presence of 'cyclized' or anhydro-vitamin A; $E^{1\%}_{1\text{ cm.}}$ 326 mμ., 52 (gross), 15 (corrected).

Sample C

(i) $E^{1\%}_{1\text{ cm.}}$ 328 mμ., 395 (gross) in *cyclo*hexane; 323 (corrected) on the basis of esterified vitamin A.

(ii) A quantity of oil (0·1696 g.) was saponified. The unsaponifiable matter was extracted as before with 50% (v/v) aqueous ethanol. The soluble portion was appropriately diluted with absolute ethanol for spectrophotometric examination: $E^{1\%}_{1\text{ cm.}}$ 325 mμ., 357 (gross), 320 (corrected).

Sample D

Four samples representative of another batch of tunny-liver oils were examined in *cyclo*hexane solution and corrected on the basis of esterified vitamin. The results were very close: $E^{1\%}_{1\text{ cm.}}$ 328 mμ., 402 (gross), 334 (corrected).

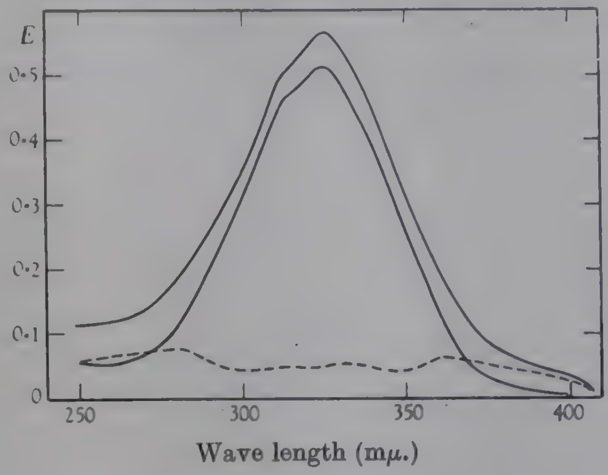

Fig. 3. Absorption spectrum of fraction soluble in 50% ethanol of the unsaponifiable fraction of tunny-liver oil (0·001323% calculated on oil used); upper curve, observed; lower curve, corrected; broken curve, irrelevant absorption. 1 cm. cell.

Discussion of the experiments on tunny-liver oils

The tunny-liver oils examined show exceptionally high values of $E^{1\%}_{1\text{ cm.}}$ 328 mμ., indicating that they contain 40–50% of vitamin A esters. The gross values of the absorption at 328 mμ. determined directly on a *cyclo*hexane solution lead, however, to a considerable over estimate of the vitamin A content because of the presence of material contributing irrelevant absorption. When the spectra are corrected using the gross readings at the fixation points (313, 328 and 338·5 mμ.) appropriate to vitamin A esters in *cyclo*hexane, the vitamin A contribution is seen to be 82–83% of the gross absorption at 328 mμ. The validity of the correction, as already explained, rests upon the assumption that irrelevant absorption is linear over the fixation range.

In order to test the assumption various methods of fractionation have been studied. Partition of the total unsaponifiable material between 83% (v/v) aqueous ethanol and light petroleum led to appreciable losses of vitamin as shown by the results on sample A. In all cases where the unsaponifiable fractions of these oils were freed from the last traces of solvent by means of steam heating in a current of nitrogen, marked losses of vitamin have occurred. It would seem that the free vitamin A as it occurs in the unsaponifiable matter of these very potent oils is more liable to oxidation than the vitamin in the corresponding fractions derived from oils of lower potency.

The method of fractionation suggested by Chevallier, Manuel & Faubert (1941) has only recently become known to us through the courtesy of M. Faubert. It consists of dispersing an oil upon silver sand as an inert support and eluting various fractions by different mixtures of ethanol and water. In trying out the method on sample B, three main fractions were obtained:

(*a*)–(*b*) fraction soluble in 70 % ethanol.
(*c*)–(*g*) fraction soluble in 80 % ethanol.
(*h*) fraction soluble in absolute ethanol.

The possibilities of the method have not yet been fully explored, but for our present purposes the use of sand is not essential.

It has been found that almost all the free vitamin A in the unsaponifiable fraction is readily soluble in 50 % aqueous ethanol. Whereas the corrected E value for the oil is 82 % of the gross value it is 92 % of the gross value for the soluble part of the unsaponifiable fraction (see sample B (iii)). Moreover, for samples B and C, the simple correction procedure applied to *cyclo*hexane solutions of the oils, gives the same final result, within very narrow limits, as the fuller fractionation procedure.

The method of fractionation with 50 % aqueous ethanol offers an alternative correction procedure to those workers who lack an accurate photoelectric spectrophotometer. It provides a means for the quantitative recovery of the vitamin A while at the same time eliminating at least half of the irrelevant absorption. It also obviates much of the risk of losing vitamin A in the final stages of preparation of unsaponifiable extracts, since after the removal of ether, the 50 % ethanol can be added at once without trying to get rid of the last traces of ethanol and water.

Conclusions from the experiments on tunny-liver oil

The correction procedure, based on the three point fixation applied to *cyclo*hexane solutions of whole oils, is justified (*a*) by fractionations of the oil and (*b*) particularly by fractionation of the unsaponifiable fraction. If facilities are lacking for the application of the full correction, the best estimate of vitamin A potency is obtained by spectrographic measurements at 325 mμ. on the 50 % ethanol extract of the unsaponifiable fraction.

The results on sample B (ii) show that with both 70 and 80 % ethanol, the amount of vitamin extracted by successive portions of solvent decreases steadily. The oil probably contains a little free vitamin A (easily soluble in 70 % ethanol). A difference is suggested between the esterified vitamin A which is soluble in 80 % ethanol from that requiring absolute ethanol, since the former solvent is progressively less effective in successive elutions despite the fact that more than half the amount of vitamin used is still available. A possible explanation of the difference is that the two fractions differ in respect of the fatty acids with which the vitamin A is esterified. Although the corrected E values for the different fractions vary from 70 to 92 % of the gross values, the addition of gross and corrected values shows that 82 % of the absorption is due to the vitamin, a result agreeing with that observed on the whole oil.

Spectrophotometric data for a range of commercial fish-liver oils

The application of the correction procedure to the determination of vitamin A in cod-liver oils has been discussed elsewhere (Morton & Stubbs, 1946). Its bearing on the conversion factor problem has been studied using the halibut-liver oil samples employed in the large-scale co-operative work organized by the Vitamin A Sub-Committee of the Accessory Food Factors Committee (Hume, 1937, 1939; Irwin, 1944). For the fully corrected $E^{1\%}_{1\,\text{cm.}}$ value a conversion factor of 1800 is appropriate (Morton & Stubbs, 1947). It is proposed here to summarize experience gained in the use of the method applied to a wide variety of vitamin A-containing materials. Table 2 shows the results obtained for a number of commercial halibut-liver oils examined in *cyclo*hexane solution.

Table 2. *Intensities of absorption shown by commercial halibut-liver oils*

	$E^{1\%}_{1\,\text{cm.}}$ 328 mμ.		Vitamin A absorption as percentage of gross absorption
Oil no.	Gross	Corrected	
1	28·8	24·2	84·2
2	54·9	47·6	86·8
3	63·4	54·0	85·2
4	39·9	34·3	86·0
5	25·6	21·1	82·4
6	29·1	24·5	84·1
7	14·6	14·0	95·9
8	98·4	95·8	97·4
9	38·0	35·7	94·0
10	26·2	23·4	89·4
11	52·2	46·2	88·6
12	58·2	49·0	84·2
13	37·5	32·1	85·6
14	29·9	28·0	93·6
15	15·0	12·6	84·0
16	25·8	24·3	94·2
			Mean 88·5

For many years the gross $E^{1\%}_{1\,\text{cm.}}$ 328 mμ. for halibut-liver oils has been multiplied by a conversion factor of 1600 in order to express the results in international units. When there is no irrelevant absorption the appropriate conversion factor is 1800. Thus only when the corrected E value is 88·9 % of the gross

Table 3. *The extent of correction needed in the spectrophotometric measurement of the vitamin A content of fish-liver oils*

Type	No. of samples	$E^{1\%}_{1\,\mathrm{cm.}}$ 328 mμ. gross	E corrected / E gross	Average conversion factor for E gross
Very rich oils	12	c. 200	0·79–0·89	1500
Rich oils	20	c. 100	0·80–0·90	1550
Halibut-liver oils	16	c. 40	0·82–0·97	1600
Shark-liver oils	15	c. 25	0·77–0·90	1510
Dogfish-liver oils	3	c. 6	0·82–0·94	1560
Hake-liver oils	6	c. 5	0·85–0·93	1610
Other liver oils	20	c. 25	0·70–0·94	1540

value will $E^{1\%}_{1\,\mathrm{cm.}}$ 328 mμ. (gross) × 1600 give the true potency. Since Table 2 shows that on the average the corrected E value for halibut-liver oil is 88·5 % of the gross value, 1600 remains the appropriate conversion factor if correction cannot for lack of the right equipment be undertaken. It is quite clear, however, that omission of correction may give rise to serious errors in some cases; the oils most contaminated with artefacts will be over valued and those least contaminated may be very significantly under valued (cf. oils 5, 7 and 8 in Table 2).

Similar results were obtained on a large number of fish-liver oils. In Table 3 many of these oils have been grouped at different potency levels rather than according to species because many commercial oils are obtained from mixed livers.

Throughout Table 3 an average empirical conversion factor between 1500 and 1600 would be justified for use with the uncorrected $E^{1\%}_{1\,\mathrm{cm.}}$ 328 mμ. value. The range of variation in the magnitude of the correction again shows, however, that over a period some oils would be over valued and others under valued, but on the average the conversion factor of 1600 has given results surprisingly near the truth.

Spectrophotometric data on commercial molecular distillates (ester concentrates)

As might have been expected, molecular distillates are easily distinguishable from ordinary commercial liver oils. The correction for irrelevant absorption is much smaller and the spectra of some samples examined are within experimental error identical in form with the standard spectrum of crystalline vitamin A acetate dissolved in *cyclo*hexane. It is significant, however, that none of the distillates so far examined require zero or negative correction as would be expected if the observed corrections were all within the experimental error of the procedure (Table 4).

Except for one sample the corrected values are over 92 % of the gross value, and in general the use of a conversion factor of 1700 is justifiable. It is not unlikely that the freshly prepared molecular distillates may have needed little or no correction. Certainly samples 7, 12 and 15 must have been representative of remarkably stable products.

Table 4. *Intensities of absorption of commercial molecular distillates in* cyclo*hexane*

Distillate no.	$E^{1\%}_{1\,\mathrm{cm.}}$ 328 mμ. Gross	$E^{1\%}_{1\,\mathrm{cm.}}$ 328 mμ. Corrected	Vitamin A absorption as percentage of gross absorption
1	100·7	92·8	92·2
2	102·2	96·6	94·5
3	102·4	95·2	93·0
4	85·8	74·2	86·5
5	85·8	82·6	96·3
6	86·8	84·0	96·8
7	87·6	86·8	99·1
8	87·8	81·9	93·3
9	86·6	80·5	93·0
10	88·2	81·2	92·1
11	86·4	81·2	94·0
12	86·8	85·4	98·4
13	87·2	81·2	93·1
14	86·0	81·2	94·4
15	85·8	84·0	97·9
			Mean 94·3

Spectrophotometric data on ling cod-liver oils

The liver oil of the ling cod (*Ophiodon elongatus*, Girard) and also the commercial oils known as 'red' and 'black' cod-liver oils are interesting in several ways. For marine fish-liver oils they are exceptionally rich in vitamin A_2 as shown by the antimony trichloride colour test. The main selective absorption in the colour test for vitamin A occurs at 617–620 mμ. whereas that characteristic of vitamin A_2 occurs at 693 mμ. In ling cod-liver oils the relative intensities of the two bands are 7–8 to 1, the latter band being the weaker. For many other liver oils from salt-water fish the corresponding ratio is 15–20 to 1. Since the potency of a commercial ling cod-liver oil may be of the order 300,000 i.u./g. the absolute amount of vitamin A_2 present is considerably higher than that normally obtainable from fresh-water fish-liver oils. After saponification and desterolation of the unsaponifiable fraction, a concentrate containing 10 % of vitamin A_2 is obtained.

From the analytical standpoint a study of such oils illustrates the possibility of further extensions of the correction for irrelevant absorption. The ultraviolet absorption curve for a ling cod-liver oil is superficially a normal vitamin A curve (Fig. 4). The criterion that in *cyclo*hexane solution

$$E_{313\ \text{m}\mu.} = E_{338\cdot 5\ \text{m}\mu.} = 6/7\ E_{328\ \text{m}\mu.}$$

is not, however, satisfied and there must therefore be irrelevant absorption. $E_{338\cdot 5\ \text{m}\mu}$ exceeds $E_{313\ \text{m}\mu.}$ in most ling cod-liver oils indicating that the slope of the irrelevant absorption is in the direction opposite to that found for most fish-liver oils. The correction procedure is, however, applicable and a reduced E value for the vitamin A contribution can readily be calculated at 328 mμ. If from the standard curve for vitamin A acetate the whole corrected vitamin A absorption curve is plotted, subtraction from the complete curve (uncorrected) of the true vitamin A contribution will give the irrelevant absorption curve. For most ling cod-liver oils the subtraction curve shows a maximum near 350 mμ. characteristic of the vitamin A_2 present in the oil, and furthermore the ratio $E_{328\ \text{m}\mu.}$ (corrected)/$E_{350\ \text{m}\mu.}$ (by subtraction) is 7·8 to 1, in very reasonable agreement in most cases with the ratio obtained from the colour test. The agreement is not strictly quantitative because there is some overlapping of the absorptions of vitamins A and A_2 in the colour test. The $E_{350\ \text{m}\mu.}$ value is the difference between two comparatively large values and as such is subject to appreciable error. There is no doubt, however, that if the position of $\lambda_{\text{max.}}$ for vitamin A_2 were not already known from data on preparations from fresh-water fish-liver oils, the subtraction procedure would have given it fairly correctly. This is of much wider significance than the present problem as it offers a means for arriving at the spectra of contaminants even though the latter may not be chemically separable from the main constituent.

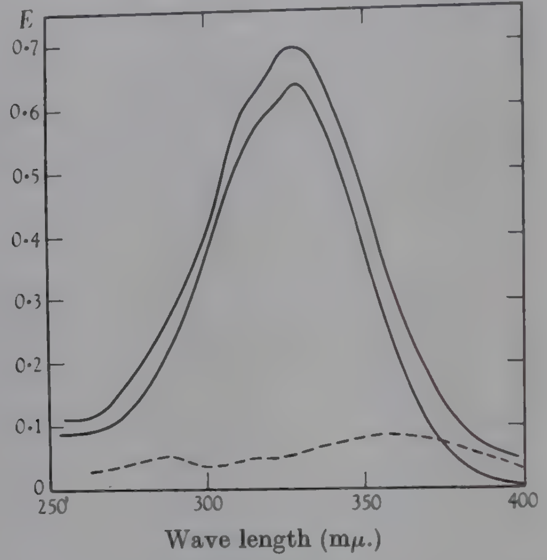

Fig. 4. Absorption spectrum of ling cod-liver oil, 0·0176% solution in *cyclo*hexane. Upper curve, observed; lower curve, corrected; broken curve, subtraction curve showing vitamin A_2 maximum. 1 cm. cell.

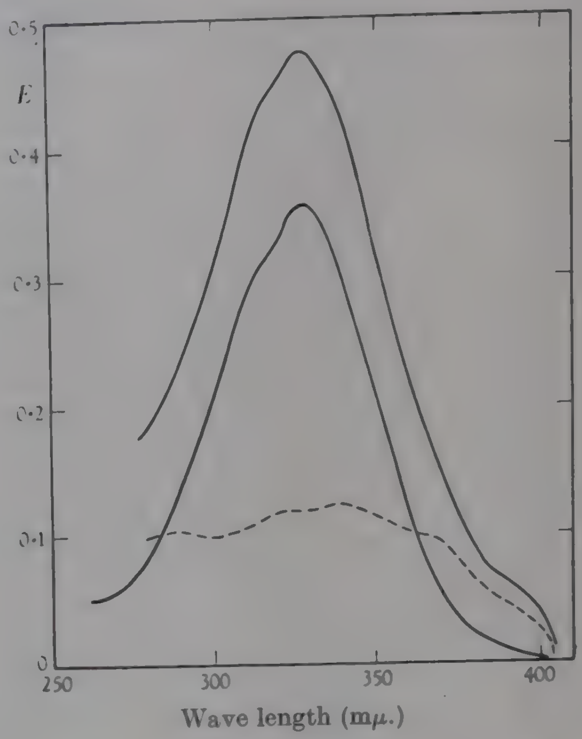

Fig. 5. Absorption spectrum of ling cod-liver oil, 0·015% solution in *cyclo*hexane. In this sample the subtraction curve shows the presence of decomposition products of vitamin A. 1 cm. cell.

Fig. 5 shows the results obtained with a ling cod-liver oil containing 'cyclized' or anhydro-vitamin A as well as vitamins A and A_2. In this case the maximum for vitamin A_2 in the subtraction curve is not so clearly shown owing to the superimposed absorption of the anhydro-product with its maxima at 350, 370 and 392 mμ. The method, however, reveals clearly the spectrum of the principal absorbing contaminant.

Data on whale-liver oils

The assay of whale-liver oils will be dealt with in a separate communication, but the problem may be referred to briefly here.

The spectra of whale-liver products vary rather widely, but in general $\lambda_{\text{max.}}$ is well on the short-wave side of 328 mμ. This is the result of very considerable irrelevant absorption due largely in refined oils to kitol with its maximum at 286 mμ. Crude oils are also contaminated with a variety of absorbing materials, especially oxidation products. The accurate assessment of vitamin A content is impossible on the uncorrected absorption, and as the correction is very variable the use of an empirically based conversion factor leads to nothing better than a first approximation. Correction by our ordinary procedure gives results in surprisingly good agreement with very much more troublesome fractionation procedures. Given a corrected $E^{1\%}_{1\,\mathrm{cm.}}$ 328 mμ. value on the oil, or corrected $E^{1\%}_{1\,\mathrm{cm.}}$ 326·5 mμ. on the unsaponifiable fraction, the use of a conversion factor of 1800 gives results which are reasonably good.

SUMMARY

1. The absorption spectra of pure vitamin A and vitamin A esters have been determined in *cyclo*hexane, ethanol and 75% (v/v) aqueous ethanol. The results are expressed in terms of the fraction of $E_{\mathrm{max.}}$ which corresponds to the absorption at different wave lengths between 250 and 400 mμ.

2. Fixation wave lengths, $\lambda_{\mathrm{max.}}$ and two others at which E is 6/7 of $E_{\mathrm{max.}}$, have been selected for vitamin A, free or esterified, in specified solvents. By their aid, irrelevant absorption in a liver oil or concentrate is readily detectable.

3. A correction procedure, previously found to be valid for cod-liver oils, has been carefully tested in relation to its validity for high-potency tunny-liver oils. The allowance for irrelevant absorption seems to be given with considerable accuracy.

4. Free vitamin A may be extracted from the total unsaponifiable fraction of fish-liver oils by 50% (v/v) aqueous ethanol. Much of the material giving rise to irrelevant absorption is left behind.

5. Experience gained in testing a wide range of fish-liver oils, including halibut, shark, hake, dogfish and many mixed liver oils shows that irrelevant absorption at 328 mμ. occurs in all, varying from 5 to 20% of the gross absorption. On the average the conversion factor of 1600 was fully justified if irrelevant absorption could not be allowed for, but the use of the factor of 1800 and of corrected E values permits the assay of individual oils with considerably improved precision.

6. Molecular distillates show from 10% to negligible irrelevant absorption. The average for the commercial samples studied is about 5%.

7. The correction procedure applied to the spectra of ling cod-liver oils enables the spectra of vitamin A_2 and anhydro-vitamin A to be obtained at least semi-quantitatively.

8. Crude and refined whale-liver oils are amenable to study by the method, and the results are an improvement upon those of any alternative procedure so far available.

Most of the fish-liver oils examined in the course of this work were obtained through the Ministry of Food.

We are indebted to Dr K. C. D. Hickman and his colleagues (Distillation Products Inc.) for specimens of crystalline vitamin A and vitamin A acetate. One of us (A.L.S.) participated in the work as holder of an I.C.I. Fellowship. The cost of the work has been defrayed from a grant for research obtained through the Scientific Adviser's Division, Ministry of Food, and the earlier studies which led up to the work were supported by the Medical Research Council.

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A High-speed Tissue Homogenizer

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This paper describes a machine designed primarily for preparing uniform dispersions or homogenates of animal tissues in aqueous media for enzyme studies. It has proved invaluable in studies of the arginase and alkaline phosphatase of tissues such as liver, kidney and mammary gland (Folley & Greenbaum, 1946, 1947), particularly in experiments involving estimation of the enzymes in tissues taken from groups of animals killed on the same day where the question of the time required for preparing tissue extracts or homogenates may be crucial.

The technique of Potter & Elvehjem (1936) for preparing homogenates of small fragments of tissue for investigation of the effects of dilution on respiratory enzyme systems was not felt to be suitable for our purpose, which was to obtain estimates of the concentration and total amount of certain water-soluble hydrolytic enzymes in whole organs of the rat. Even though the apparatus of Potter & Elvehjem might well be built on a scale sufficient for 50–60 ml. of fluid, it seemed clear that our work required a relatively robust, yet 'precision' machine, easy to use and capable of dealing rapidly and reproducibly with fair-sized masses of perhaps tougher tissue than one might expect could be easily and routinely handled with the homogenizer of Potter & Elvehjem.

How far our apparatus will be applicable to making tissue dispersions for the study of other tissue enzyme systems and perhaps hormones remains to be seen. Preliminary trials suggest that it may prove useful for homogenizing plant materials, textile fibres, etc.

DESCRIPTION OF HOMOGENIZER

The machine is illustrated in Figs. 1 and 2. It incorporates the principle of rotating knives, a principle which had already been embodied in the Waring Blendor. The homogenization is carried out in the Pyrex glass vessel *A*, consisting of a tube blown at one end into a bulb of 100 ml. capacity, the walls of which are fluted in such a way that the cross section through its greatest diameter is as shown in the lower drawing of Fig. 2. This glass vessel is held firmly and quite centrally inside a cylindrical metal container *B* by the accurately turned fibre collar *C* and the rubber ring *D*, the centre of which is shaped to the bottom of the bulb. Three phosphor-bronze springs *E*, fixed to the inner wall of the container and spaced equidistantly, help to centre the bulb and hold it firmly in position. Vibration during running is minimized by rubber studs inserted into the inner and outer edges of the fibre collar, and projecting slightly so that they press against the outer surface of the glass tube and the inner surface of the metal container respectively.

The tissue to be homogenized is weighed, cut into pieces about the size of a pea with scissors, and placed in the glass vessel together with a suitable volume of the chosen dispersion fluid (saline, Ringer, etc.). The machine works best with a total volume of tissue and fluid of about 50 ml., but in exceptional cases volumes as small as 30 ml. or as high as 60 ml. have been used. Larger volumes cannot be used with vessels of the present dimensions because of the danger of splashing when the machine is running at speed.

The container assembly is put together as shown in the sectional elevation in Fig. 2, and inserted through the collar *F* which it fits accurately. A small dowel on the outside of the container passes up through a slot in the inner face of the collar, the width of which undergoes a slight but gradual increase for about 2 in. in an anti-clockwise direction from the slot, so that on turning the container through about an eighth of a circle with the dowel resting on the top edge of the collar, the flange at the base of the container is brought up tight against the bottom of the collar. The set screw *G* is then tightened to hold the container immobile during the run. The inside of the container is strengthened at the bottom by a metal ring *H* to withstand the pressure of the set screw.

The Universal motor *J* revolves at 12,000–15,000 r.p.m. To its shaft is directly coupled a stainless steel spindle carrying at its lower end four accurately balanced stainless steel knives *K*, two of which are horizontal and two project upwards and downwards respectively at an angle of 45°. Thus a considerable proportion of the interior space of the bulb is swept by the revolving knives, which are of such a length as to pass easily down the neck of the dispersion vessel. The length of the spindle is such that when the apparatus is assembled the two horizontal knives lie below the plane of the greatest diameter of the bulb (*A—A* in Fig. 2).

A stainless steel sleeve *L* attached to the spindle overlaps the tapered end of the motor casing *M*, thus preventing oil from the motor bearings creeping down the shaft. At the bottom of the sleeve is fixed a fibre disk *N*, of diameter slightly less than the internal diameter of the neck of the dispersion vessel, to prevent loss of fluid due to splashing. This, however, does not occur if due precautions are taken in starting up.

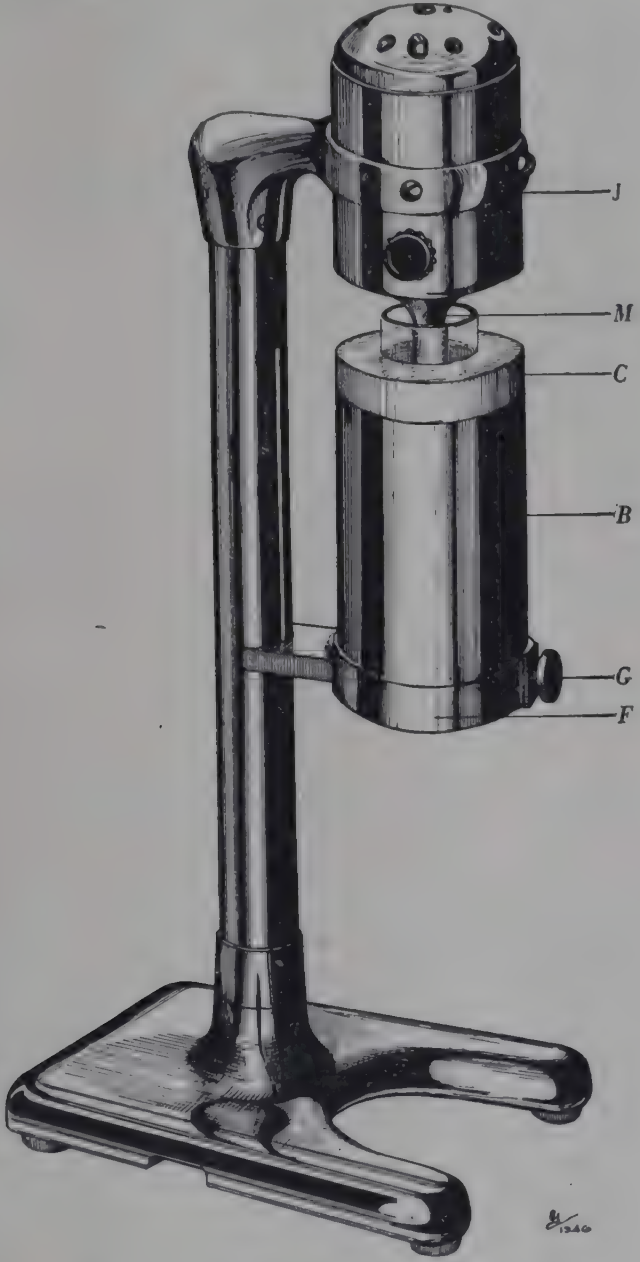
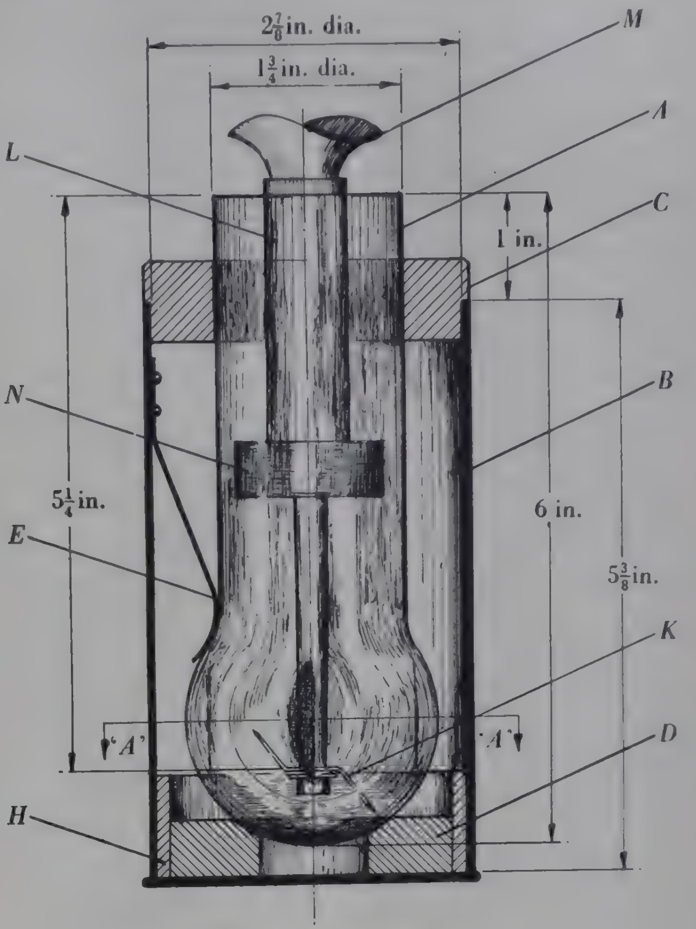
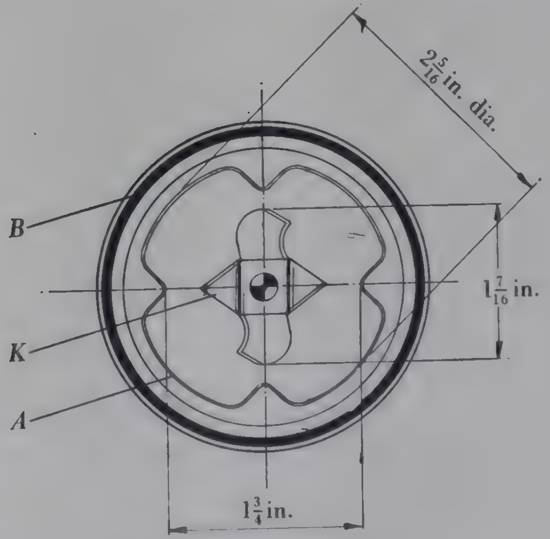


Fig 1. Perspective view of homogenizer.

A suitable sliding resistance is used for starting, and in order to prevent splashing, it is necessary to start slowly, and run at a reduced speed for some seconds until it is obvious from the sound coming from the interior of the dispersion vessel that the lumps of tissue have been largely broken down. Only then is the motor taken up to full speed. If this precaution is not observed the whirling mass of tissue temporarily conglomerated round the knives would tend to force the fluid out of the dispersion vessel. The fluted shape of the bulb is necessary to offer resistance to the rotation of the fluid and to throw it and the tissue into the knives.



Sectional elevation



Section '*A—A*'

Fig. 2. Sectional scale drawings showing details of dispersion vessel assembly.

Tissues like liver and kidney are uniformly dispersed with the greatest of ease. Stained smears of such homogenates consisted merely of amorphous material quite unrecognizable as belonging to the organ in question. There were scattered deformed nuclei present but no clear cellular outlines. Occasionally somewhat larger irregular masses could be recognized as containing connective tissue and fragments of blood vessels. The homogenates remain stable for considerable periods and can be pipetted with ordinary pipettes without previous filtration; some sedimentation slowly occurs on standing over some hours, but this heavier material can be redistributed by shaking to give relatively stable homogeneous fluids from which accurate samples can be taken. Lactating mammary tissue also gives good homogenates in 5 min., but these contain small strands of fibrous-connective tissue which should be removed by filtration through glass wool before pipetting. These strands are more prominent in homogenates of involuting mammary gland, which is much more generously interlaced with fibrous-connective stroma, but it is believed that all of the glandular tissue is comminuted. Even with tough tissues like rat's skin, tendon or the cervical region of the uterus, dispersion for 5 min. in this apparatus produces a surprising degree of comminution. These and many other tissues, which the machine can handle with ease, present a formidable proposition when ground with sand in a pestle and mortar.

The contents of the dispersion vessel tend to warm up during homogenization, and if necessary can be cooled by packing the space between the dispersion vessel and the container with ice. Alternatively, it is easy to maintain the tissue at body temperature from the time of removal from the animal until the start of the enzyme determination.

SUMMARY

A machine for the easy preparation of uniform tissue homogenates for enzyme studies is described.

Our best thanks are due to Mr K. C. Richardson for histological examination of tissue homogenates. We are also indebted to Mr K. W. Gladwell of Messrs Nelco Ltd., Shalford, Guildford, for invaluable help with the production of the machine, and to Mr C. Machin for the drawings.

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Component Acids of the Lipids of Human Tubercle Bacilli. Part 1

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Investigations by Anderson and his associates on the chemistry of the lipids of various acid-fast bacilli demonstrated the presence in the bacterial lipids of certain new and specific saturated branched-chain fatty acids of high molecular weight. The acids gave ether-soluble lead salts, and this property was utilized for their isolation from the original mixtures; the separation of the individual acids was accomplished by fractional distillation of the corresponding methyl esters.

Tuberculostearic acid and phthioic acid, isolated by the above procedure from the acetone-soluble fat of the human type tubercle bacillus, strain H-37, appeared to be characteristic components of the lipids of human tubercle bacilli, although the composition of the lipids was found to vary from strain to strain (cf. Crowder, Stodola, Pangborn & Anderson, 1936). More recently studies of cell residues from the preparation of the purified tuberculin protein PPD, derived from an unidentified strain of the human tubercle bacillus, indicated the presence of various branched-chain fatty acids which had not been encountered in previous investigations (Peck & Anderson, 1941; Edens, Creighton & Anderson, 1944; Ginger & Anderson, 1944). According to the last-mentioned paper a dextrorotatory ester fraction, which resembled methyl phthioate (cf. Spielman & Anderson, 1935–6), gave on extended fractionation four fractions varying in composition and in the magnitude of optical rotation.

Investigations in this laboratory (Polgar & Robinson, 1945) led to the synthesis of a number of methyl-substituted long-chain acids, of which 3:13:19-trimethyltricosanoic acid exhibited properties resembling those of phthioic acid. Biological studies by Dr C. E. Coulthard, of the Research Department of Boots Pure Drug Co. Ltd., and Dr J. Ungar, of Glaxo Laboratories Ltd., indicated that the synthetic acid produces in experimental animals lesions similar to the tubercle, the characteristic lesion of tuberculosis; the activity of the acid in this respect seemed about equal to that reported for phthioic acid.

PRESENT INVESTIGATION

Characterization of fatty acids through semicarbazones of their acetol esters

In view of the above position, further studies seemed desirable. On searching for derivatives, which would facilitate the characterization and purification of such acids, the acetol esters, $R.CO_2CH_2.CO.CH_3$, were found to provide a suitable starting point. Semicarbazones of such esters have been described (Locquin, 1904). In the present investigation, acetol esters of various acids were obtained in almost quantitative yields by refluxing an alkali salt of the acid in ethanolic solution with an excess of chloroacetone. On treatment with semicarbazide, the acetol esters of palmitic and stearic acids gave *semicarbazones* (m.p. 111·5 and 112·5–113·5°, respectively), which were very sparingly soluble in cold ethanol, whereas the *semicarbazones* of acetol 13:16-dimethyltricosanoate and acetol 13:17:21-trimethyldocosanoate (m.p. 67–68° and 70–71°, respectively), obtained in the same way, were comparatively soluble in this solvent, and the semicarbazone of acetol 3:13:19-trimethyltricosanoate was an easily soluble oil. A less soluble and higher melting derivative of the last-mentioned ester could be obtained by treatment with 2:4-dinitrophenylsemicarbazide (McVeigh & Rose, 1945); the resulting *dinitrophenylsemicarbazone* (m.p. 137–138°) was a pale yellow substance which also seemed well suited for application to chromatographic analysis.

The acids could be easily recovered from the foregoing derivatives by alkaline or acid hydrolysis. The acids obtained on hydrolysis of the dinitrophenylsemicarbazones were contaminated with nitro compounds derived from the reagent, but the latter could be readily removed by treating the products with tin and hydrochloric acid, the amines formed by the reduction being easily soluble in the aqueous medium.

Fractionation of fatty acids from human tubercle bacilli

These findings suggested that it might be possible to apply the fractional crystallization of such derivatives to the mixed acids of the tuberculolipids as a step towards isolating the individual acids. The lipids of the residues of human tubercle bacilli were extracted by known procedures and hydrolyzed with ethanolic potassium hydroxide. The acidified product, after removing incompletely hydrolyzed waxy substances by precipitation from an ethereal solution with methanol, was subjected to the scheme of separation described below.

As a preliminary to the application of the fractional crystallization processes, the whole mixture of fatty acids was separated, by fractional distillation of the corresponding methyl esters, into three fractions; each fraction was then converted in the manner already described into acetol esters and thence into semicarbazones. Crystallization of the latter from ethanol gave a sharp separation from very sparingly soluble products which were mainly derived from normal-chain acids. The remaining mixtures were further separated by fractional crystallization and the products so obtained eventually subjected to alkaline hydrolysis to regenerate the corresponding acids.

The ethanolic mother liquors remaining from the above crystallizations contained easily soluble substances of low melting points. After regeneration of the acids from the isolated products, the resulting mixture of acids was oxidized with hydrogen peroxide in formic acid (cf. Swern, Billen, Findley & Scanlan, 1945), the purpose of this operation being to convert unsaturated substances into hydroxylated derivatives. The free hydroxy acids were then isolated by treatment with light petroleum in which they were very sparingly soluble. The remaining petroleum solution contained saturated branched-chain acids which were subjected to further separations by conversion into acetol esters, and then into 2:4-dinitrophenylsemicarbazones. The latter were crystallized from various solvents, and eventually the corresponding acids recovered by hydrolysis of the products. On applying the above scheme to the three methyl-ester fractions obtained from the crude mixture of fatty acids, the lowest ester fraction (b.p. 120–130°/0·5 mm.) appeared to consist mainly of methyl palmitate, and has not been investigated further.

The intermediate fraction (b.p. about 140–160°/0·7 mm.) yielded, on conversion of the acids into acetol esters and thence into semicarbazones, among other products a *semicarbazone* melting at 72–73° which seemed to be derived from a C_{19} acid, in the following referred to as acid A. The regenerated and purified acid was a low-melting solid, m.p. 23–24°; for comparison with tuberculostearic acid it has been further characterized as its amide, m.p. 78–79°, and by the preparation of the tribromoanilide, m.p. 97·5–98°. The melting points of these derivatives correspond within a few degrees to those recorded for the amide and tribromoanilide of tuberculostearic acid (76–77 and 93–94°, respectively; cf. Spielman, 1934), but there is a discrepancy of about 12° between the melting point of the free acids (23–24° as against 10–11°). It may be recalled that Spielman, ascribing to tuberculostearic acid the structure of 10-methylstearic acid, found a similar divergency between the melting point of tuberculostearic acid (10–11°) and that of the synthetic acid (20–21°), whereas the amides and tribromoanilides had identical melting points. Spielman considered the possibility that tuberculostearic acid may contain, in addition to 10-methylstearic acid, another acid of similar structure, such as 9-methylstearic acid, which could have escaped detection. However, he preferred the explanation that the difference in the melting points of the acids was due to the presence of an asymmetric carbon atom.

In the present experiments, the substances remaining in the mother liquors of the above semicarbazone derived from acid A were hydrolyzed to recover the corresponding acids, and the latter oxidized with hydrogen peroxide in formic acid; after removing hydroxy acids, apparently mainly consisting of dihydroxystearic acid (formed by oxidation of oleic acid), an oil remained which seemed to contain a saturated branched-chain acid different from acid A. It will be necessary to investigate this substance more closely, in order to clarify the position.

The present work was mainly concentrated upon the higher methyl-ester fraction (b.p. about 170–215°/0·7 mm.). This yielded, by application of the same processes, three saturated branched-chain acids, in the following referred to as B 1, B 2 and B 3; besides, a dihydroxy acid, B 4, which was obtained by the use of the hydrogen peroxide-formic acid procedure. Acid B1 was isolated as its *acetol-ester semicarbazone*, m.p 44–45°. The regenerated acid, which had m.p. 25° and $[\alpha]_D + 4{\cdot}8°$, was further characterized by a series of derivatives, namely, as its *amide*, m.p. 46°, *tribromoanilide*, m.p. 64–65°, and p-*bromophenacyl* ester, m.p. 33–34°. The analytical values indicate $C_{30}H_{60}O_2$ or its near homologues as possible formulae for the parent acid. It may be mentioned in this connexion that a C_{30} acid, designated mycocerosic acid, has been isolated by Anderson and his associates from the waxes of human tubercle bacilli (cf. Ginger & Anderson, 1945), but this acid was laevorotatory, and its *p*-bromophenacyl ester had m.p. 47–48°.

Acid B 2 was obtained as its *acetol-ester semicarbazone*, m.p. 81–82°, from the mother liquors of the semicarbazone derived from acid B 1. The *acid* had m.p. 22° and was also optically active, $[\alpha]_D + 11{\cdot}7°$. It formed an *amide*, m.p. 44°, and a *tribromoanilide*, m.p. 47°. From the analytical figures for these derivatives, the acid may have the composition $C_{27}H_{54}O_2$ or $C_{28}H_{56}O_2$. The acetol-ester semicarbazone derived from acid B 3 was liquid at room temperature and very easily soluble in organic solvents; it remained in the mother liquors which resulted from the fractional crystallizations of the semicarbazones derived from the acids B 1 and B 2. The recovered acid, after removing an unsaturated component (B 4, discussed below) by the hydrogen peroxide-formic acid process, and admixed low-boiling acids by fractionation of the methyl esters, was converted into its acetol ester, and thence into the 2:4-*dinitrophenylsemicarbazone*. The latter had m.p. 153–154·5°, and the analytical figures agreed with those required for phthioic acid, $C_{26}H_{52}O_2$, as the parent acid. The regenerated acid solidified at 0° but melted at room temperature; its specific rotation was about +11°. The amide showed very poor crystallizing properties, and the tribromoanilide was obtained as a low-melting solid which liquefied at room temperature. The acid obviously requires further purification. Kuhn-Roth determination of C-methyl gave 3·4 mol. of acetic acid from acids B 1 and B 3, and 2·9 mol. from acid B 2, as compared with 2·1 mol. of acetic acid in case of acid A. Assuming that the last is a monomethylstearic acid, comparison of the above values would indicate the presence of possibly four terminal methyl groups in case of the acids B 1 and B 3, and three such groups in acid B 2.

Preliminary studies of monolayers of the acids B 1, B 2 and B 3 indicate that only acid B 3 exhibits properties like those characteristic for phthioic acid (cf. Stenhagen & Ställberg, 1941), collapsing on compression at about 40 A.^{2}; acids B 1 and B 2 appear to give unstable films continually collapsing on compression.

It remains to describe briefly the *dihydroxy acid* B 4 which has been mentioned above in connexion with the isolation of acid B 3. It had m.p. 69–70°, and the analytical data were in agreement with a composition $C_{26}H_{52}O_4$ (or near homologues). Oxidation with chromic acid by the Kuhn-Roth method gave 1·6 mol. acetic acid, indicating the presence of two terminal methyl groups.

The results of this preliminary study indicate that a certain separation of the mixture of acids can be achieved by employing the scheme described. However, it will be necessary, in larger-scale experiments, to supplement the above processes by efficient ester fractionations.

For comparison with the above results, the same scheme of separation has been recently applied by the present author to a mixture of acids from the acetone-soluble fat of *Mycobacterium phlei*, a quantity of which was kindly provided by Imperial Chemical Industries Ltd., Dyestuffs Division. The results of these studies will be published in a later communication, but they may be anticipated by stating that the saturated branched-chain acids of higher molecular weight produced by this non-pathogenic member of the acid-fast group appear to be entirely different from those of the human tubercle bacillus.

EXPERIMENTAL

Analyses, including C-methyl determinations, are by Drs Weiler and Strauss, Oxford.

Acetol esters. The following preparation of *acetol palmitate* is typical: A solution of palmitic acid (0·5 g.) in ethanol

(10 ml.) was neutralized with ethanolic KOH, using phenolphthalein as indicator; chloroacetone (0·5 g.) was then added and the mixture refluxed for 2 hr. Next day, water was added and the product collected by ether. The ethereal extract was washed thoroughly with water, and the residue remaining after evaporation of the ether solution heated at 100° under reduced pressure in order to remove completely any unreacted chloroacetone. The resulting ester (yield, practically quantitative) crystallized from ethanol in the form of needles, m.p. 50°. (Found: C, 73·2; H, 11·6. $C_{19}H_{36}O_3$ requires C, 73·1; H, 11·5%.) On treatment with semicarbazide acetate in ethanolic solution it gave a *semicarbazone*, m.p. 111·5° (from ethanol). (Found: C, 65·3, 65·5; H, 10·2, 10·6. $C_{20}H_{39}O_3N_3$ requires C, 65·0; H, 10·6%.)

The following *semicarbazones* were similarly prepared: *acetol stearate semicarbazone*, m.p. 112·5–113·5° (Found: C, 66·7; H, 10·8. $C_{22}H_{43}O_3N_3$ requires C, 66·5; H, 10·8%); *acetol* 13:16-*dimethyltricosanoate semicarbazone*, m.p. 67–68° (Found: C, 70·0; H, 11·3. $C_{29}H_{57}O_3N_3$ requires C, 70·3; H, 11·5%); *acetol* 13:17:21-*trimethyldocosanoate semicarbazone*, m.p. 70–71° (Found: C, 70·25; H, 11·3. $C_{29}H_{57}O_3N_3$ requires C, 70·3; H, 11·5%).

Acetol 3:13:19-trimethyltricosanoate, on treatment with semicarbazide acetate, gave an oil. Its 2:4-*dinitrophenylsemicarbazone*, obtained by short refluxing of an ethanolic solution of the ester with the calculated amount of 2:4-dinitrophenylsemicarbazide, was a pale yellow powder, m.p. 137–138°, after recrystallization successively from ethanol and benzene-ethanol. (Found; C, 64·2; H, 8·75. $C_{36}H_{61}O_7N_5$ requires C, 64·0; H, 9·0%.)

Extraction of the lipids of tubercle bacilli. The bacterial cells used for this investigation were residues from the manufacture of tuberculin (PPD), kindly provided by the Veterinary Laboratory, Weybridge, of the Ministry of Agriculture and Fisheries. They were derived from three human strains of *Myco. tuberculosis*, designated 'mixed P.N., D.T. and C', which were grown on a synthetic medium (glycerol, glucose, asparagine) for 10 weeks, and then killed by the action of steam. The bacterial cells were extracted by successive treatment with ethanol, ethanol-ether (1 : 1), and ether-chloroform (1 : 1) at room temperature. The solutions were decanted, filtered from the bacterial cells, and evaporated under reduced pressure. The residual products were then combined and extracted with ether.

Isolation of the mixed fatty acids. The above ethereal extract was evaporated, and the residue (about 270 g.) hydrolyzed by refluxing for 2·5 hr. with a solution of KOH (40 g.) in water (40 ml.) and ethanol (120 ml.). Under these conditions the fats are hydrolyzed, but the waxes incompletely or only to a slight extent. After addition of water and acidification, the product was collected by means of ether, and waxy substances separated by precipitation of the ethereal solution with methanol. The solution remaining after removal of the precipitate was evaporated and the residue (80 g.) converted into methyl esters by refluxing for 3 hr. with 10% (w/v) methanolic H_2SO_4.

Fractionation of the methyl esters. The following fractions were collected: a low-boiling fraction, b.p. 120–130°/0·5 mm. (7·4 g.); a middle fraction, b.p. 140–160°/0·7 mm. (41 g.); and a high-boiling fraction, b.p. 170–215°/0·7 mm. (8·3 g.).

The low-boiling fraction. This was only tentatively tested. A small sample was hydrolyzed and the acid converted into its acetol ester, and thence into the semicarbazone in the manner already described. The semicarbazone was obtained as a white crystalline powder, m.p. 110–110·5° after crystallization from ethanol. (Found: C, 64·5; H, 10·5. $C_{20}H_{39}O_3N_3$ requires C, 65·0; H, 10·6%.) There was no depression on admixture with the semicarbazone of acetol palmitate; however, this is not of great significance, as the melting points of several acetol-ester semicarbazones derived from normal-chain acids lie close together and are not depressed on admixture. Hydrolysis of the above semicarbazone gave an acid, m.p. 61° alone or mixed with palmitic acid.

The middle fraction. The ester (41 g.) was hydrolyzed by refluxing with ethanolic KOH for 3 hr. Water was then added, and the alkaline solution extracted with ether; a small quantity (0·4 g.) of a non-acidic product was thus obtained. Acidification of the alkaline solution gave a mixture of acids which were isolated by ether extraction. After removal of the ether, the product was warmed with ethanol (50 ml.) and the solution kept for 3 hr. It deposited 5 g. of a solid, m.p. 52–53°, probably mainly a mixture of normal-chain acids.

The remaining solution was neutralized with ethanolic KOH; a large excess of chloroacetone (17 g.) was then added and the mixture refluxed for 3 hr. The product, isolated in the manner already described, was dissolved in ethanol (200 ml.) and brought to reaction with semicarbazide acetate (from 16 g. of semicarbazide hydrochloride and 24 g. of sodium acetate in 20 ml. of water). Next day the precipitated mixture of semicarbazones was filtered off; recrystallization from ethanol gave a sharp separation from a very sparingly soluble product (12 g.), m.p. 98–100°, probably mainly derived from normal-chain acids. On addition of water to the mother liquors a semicarbazone, m.p. 68° (sintering at 63°), separated; after two further crystallizations from aqueous ethanol the product was obtained as minute needles (5 g.), m.p. 72–73°. (Found: C, 66·8, 66·7; H, 10·7, 10·8. $C_{23}H_{45}O_3N_3$ requires C, 67·2; H, 10·95%.) The corresponding acid, regenerated from the semicarbazone by hydrolysis with ethanolic KOH, was a mobile oil. To remove admixed unsaturated substances, the product was heated with formic acid (98%; 10 ml.) and hydrogen peroxide (30%; 2·5 g.) at 60° (bath temperature) with stirring for 1 hr. Next day water was added and the product, isolated by ether extraction, refluxed with ethanolic KOH, in order to hydrolyze the formoxy compounds which were formed in the foregoing process. On keeping the free acid with light petroleum (b.p. 40–60°) a small quantity of a substance, m.p. 78°, was deposited. The remaining solution was then evaporated and the residual product esterified by means of methanolic H_2SO_4. The methyl ester distilled as a colourless liquid (2·5 g.), b.p. 157–160°/1 mm., $n_D^{12·5°}$ 1·4480. Hydrolysis of the ester gave the corresponding acid (A), m.p. 23–24°, C-methyl, 10·5%. It gave an amide, m.p. 78–79° (from methanol). (Found: C, 76·5; H, 12·8. Calc. for $C_{19}H_{39}ON$: C, 76·8; H, 13·1%.) The tribromoanilide crystallized from ethanol in needles, m.p. 97·5–98°. (Found: C, 48·8; H, 6·3. Calc. for $C_{25}H_{40}ONBr_3$: C, 49·2; H, 6·6%.)

On concentrating the mother liquors of the semicarbazones described in the preceding paragraph an oil separated. Water was added, and the product isolated with ether. It was hydrolyzed by means of ethanolic KOH, and the acidified product extracted with ether. The product remaining after evaporation of the ethereal extract was then oxidized by hydrogen peroxide (30%; 7 g.) in formic acid (98%; 25 ml.) at 50° (bath temperature) for 1 hr., while

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stirring the mixture. Next day water was added and the product, isolated by ether extraction, refluxed with ethanolic KOH for 2 hr. After isolation of the acidified product (ether extraction), it was treated with light petroleum (b.p. 40–60°). The substance (5 g.), which separated from the petroleum solution, had m.p. 78°, raised to 93·5° after two crystallizations from 95% ethanol. (Found: C, 68·3; H, 11·05. Calc. for $C_{18}H_{36}O_4$: C, 68·4; H, 11·4%.) From its melting point and analytical values this substance appears to be 9:10-dihydroxystearic acid (m.p. 95°), arising from the oxidation of oleic acid.

The remaining petroleum solution seemed to contain a saturated branched-chain acid; this will be investigated more closely when opportunity permits.

The high-boiling fraction. The ester (8·3 g.) was hydrolyzed with ethanolic KOH. On attempting to remove non-acidic products, by ether extraction of the alkaline solution, emulsions resulted; therefore, the solution was acidified and the product collected with ether. The ethereal solution was then evaporated and the residue kept with ethanol (20 ml.); a crystalline product (0·5 g.) of m.p. 82–83° separated, probably mainly normal-chain acid. (Found: C, 78·0; H, 12·7. Calc. for $C_{24}H_{48}O_2$: C, 78·3; H, 13·0%.) The remaining ethanolic solution was neutralized with sodium ethoxide (0·5 g. of sodium in 10 ml. of ethanol); an excess of calcium chloride in ethanolic solution was then added and after keeping the mixture for several hours, the precipitated calcium salt filtered off. This was decomposed with HCl, and the liberated acid isolated with ether (weight of substance, 4·8 g.). The ethanolic mother liquors of the calcium salt were evaporated and the residue, after treatment with HCl, isolated by ether extraction. The ethereal solution, after washing it first with aqueous KOH and then thoroughly with water, yielded on evaporation 0·8 g. of non-acidic material. The combined alkaline and aqueous washings were acidified and extracted with ether; evaporation of the ether left 1·7 g. of acidic substance which was combined with the material obtained from the precipitated calcium salt.

The above acid (6·5 g.) was neutralized with a solution of sodium ethoxide in ethanol (from 0·5 g. of sodium in 10 ml. of ethanol), and the product brought to reaction with an excess of chloroacetone (7 g.) by refluxing the mixture for 5 hr. The resulting acetol ester was then treated in ethanolic solution with semicarbazide acetate in the usual manner. On crystallization of the crude semicarbazone from ethanol a very sparingly soluble substance (0·22 g.) having m.p. 103–104·5°, probably derived from normal-chain acids, separated first. From the mother liquors two additional *semicarbazones* were obtained by fractional crystallization; one of these (B1, 1 g.) had m.p. 44–45°. (Found: C, 72·4, 72·1; H, 11·6, 11·7. $C_{34}H_{67}O_3N_3$ requires C, 72·2; H, 11·9%), and the other (B 2, 0·45 g.), which separated on concentration of the combined ethanolic mother liquors of the foregoing semicarbazone, melted at 81–82°. (Found: C, 71·55; H, 11·4. $C_{32}H_{63}O_3N_3$ requires C, 71·5; H, 11·7%.) Further concentration of the mother liquors of this product gave an oil which was isolated by the addition of water, followed by ether extraction. It was then hydrolyzed with ethanolic KOH and the recovered acid (about 4·5 g.) subjected to a repetition of the above processes (conversion into acetol esters, and thence into semicarbazones). A small quantity (0·3 g.) of solid semicarbazones was obtained which on crystallization from ethanol yielded further amounts of the semicarbazones B 1 and B 2; the main product was again an oil. The latter was subjected to the following processes.

The recovered acid was treated with hydrogen peroxide (30%; 2 g.) in formic acid (98%; 12 ml.) at 50° for 1·5 hr. in the manner previously described. After hydrolyzing the resulting formoxy compounds by means of ethanolic KOH, the free acid (isolated by ether extraction) was refluxed with light petroleum (b.p. 40–60°) for a few minutes, and the mixture allowed to stand overnight. It deposited a *substance* (acid B 4, about 0·1 g.), m.p. 69–70° (after recrystallization from aqueous ethanol). (Found: C, 72·7; H, 11·9. $C_{26}H_{52}O_4$ requires C, 72·9; H, 12·15%); C-methyl, 5·5%.

The petroleum solution was then evaporated, and the residual product converted into its methyl ester by refluxing with 10% (w/v) methanolic H_2SO_4 for 3 hr. On fractionation of the ester a fore run (0·85 g.) distilled below 165°/0·7 mm.; the remainder distilled mainly at about 190–210°/0·7 mm. as a viscous oil (2·05 g.), $n_D^{17.5^\circ}$ 1·4620, $[\alpha]_D + 8{\cdot}2^\circ$ (in ether; $c = 11$). The product was hydrolyzed with ethanolic KOH, and the resulting acid converted by the usual procedure into its acetol ester. A solution of this ester in ethanol (40 ml.) was refluxed with 2:4-dinitrophenylsemicarbazide (1·4 g.) for about 10 min., and the solution then filtered hot. Next day the precipitated product was collected and crystallized successively from ethanol and benzene-ethanol. A *dinitrophenylsemicarbazone*, m.p. 153–154·5° was thus obtained as the main product (1 g.) which on hydrolysis, as will be described below, gave acid B3. (Found: C, 63·7; H, 8·6; N, 10·4. $C_{36}H_{61}O_7N_5$ requires C, 64·0; H, 9·0; N, 10·4%.)

*Acid B*1. This was obtained from the above semicarbazone, m.p. 44–45°, by hydrolysis with ethanolic KOH. The *acid* distilled at 210–220°/0·05 mm. as a viscous oil (0·76 g.) which quickly solidified and had m.p. 25°; $[\alpha]_D + 4{\cdot}8^\circ$ (in ether; $c = 15{\cdot}2$); C-methyl, 11·7%. The acid was saturated, and gave an *amide*, m.p. 46° (from methanol) (Found: N, 3·1. $C_{30}H_{61}ON$ requires N, 3·1%.) Its *tribromoanilide*, after three crystallizations from ethanol, melted at 64–65° (sintering at about 60°). (Found: C, 57·0; H, 7·9. $C_{36}H_{62}ONBr_3$ requires C, 56·6; H, 8·1%.) The p-*bromophenacyl* ester had m.p. 33–34° (sintering at 30°) after repeated crystallizations from ethanol. (Found: C, 69·9; H, 9·6. $C_{38}H_{65}O_3Br$ requires C, 70·3; H, 10·0%.)

*Acid B*2. Hydrolysis of the semicarbazone previously described, m.p. 81–82°, in the usual manner afforded the crude *acid* which distilled from a bath at 210–220°/0·05 mm. as a viscous oil (0·32 g.). It quickly solidified and had m.p. 22°, $[\alpha]_D + 11{\cdot}7^\circ$ (in ether; $c = 6{\cdot}5$), C-methyl, 10·5%; the acid was saturated. It gave an *amide*, m.p. 44° (from methanol). (Found: N, 3·25. $C_{28}H_{57}ON$ requires N, 3·3%.) The *tribromoanilide* crystallized from ethanol as short needles, m.p. 47°. (Found: C, 55·0, 55·3; H, 7·6, 7·5. $C_{33}H_{56}ONBr_3$ requires C, 54·9; H, 7·8%. $C_{34}H_{58}ONBr_3$ requires C, 55·5; H, 7·9%.)

*Acid B*3. The isolation of the acetol-ester dinitrophenylsemicarbazone derived from this acid has been outlined above. Hydrolysis with a mixture of sulphuric acid, acetic acid and water (5 ml. of each), by refluxing the mixture for 3 hr., afforded the crude acid which was contaminated with nitro compounds. After isolating the product by ether extraction, it was heated with tin (1·2 g.) and HCl (10 ml. of conc. acid and 5 ml. of water) on a steam bath for 1 hr. Water was then added, and the acid isolated by ether. It distilled at 200–210°/0·05 mm. as a pale yellow oil which solidified on cooling with ice, and melted at about 20°; $[\alpha]_D + 11\cdot 1^\circ$ (in ether; $c = 11\cdot 1$); C-methyl, 12·8%. The amide was obtained as a low-melting solid which was very soluble in organic solvents; the tribromoanilide separated from aqueous methanol as an oil.

SUMMARY

1. A scheme of separating mixtures of the component fatty acids from the lipids of human tubercle bacilli is described. The scheme, which may be found of value in other connexions, depends upon the fractional crystallization of derivatives, obtainable by converting the acids into acetol esters, $R.CO_2CH_2.CO.CH_3$, and treating the latter with an appropriate reagent for ketones, such as semicarbazide or 2:4-dinitrophenylsemicarbazide.

2. The acids isolated by this process include a saturated C_{19}-acid, resembling tuberculostearic acid in respect of the melting points of derivatives; however, the melting points of the free acids are found to differ by about 12°, and the available evidence seems to indicate the presence of an additional acid of similar composition, having a lower melting-point.

3. From a mixture of the acids of higher molecular weight three dextrorotatory products are obtained by the above procedure; of these, two appear to contain four terminal methyl groups, and the third, three such groups.

4. The presence of an unsaturated branched-chain acid of high molecular weight is indicated by the isolation of its oxidation product; for the oxidation hydrogen peroxide in formic acid has been employed.

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The Determination of Glutathione and other Substances Contained in Yeast

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In the course of an investigation of the action of glutathione on the growth of tissue cells *in vitro* (Astrup & Fischer, 1946) we made some determinations of the amount of glutathione present in Danish yeasts. During these determinations some difficulties were encountered which are of importance also for the determination of other substances contained in yeast and which, as far as we could find, have not previously been described.

METHODS

Glutathione was measured colorimetrically with nitroprusside (Fujita & Numata, 1939; Numata, 1940), using, however, 2N-NH_3 instead of a N-solution for developing the colour, and a standard curve made under identical conditions for comparison. Reduction of GSSG to GSH was carried out electrolytically after Dohan & Woodward (1939). Phosphorus was estimated according to Fiske & Subbarow (1925), using a Pulfrich photometer (Teorell, 1931). Glutathione was prepared by the method of Pirie (1930).

RESULTS

When metaphosphoric acid was used for extracting GSH from yeast no definite results could be reached. Thus, Fig. 1 shows a curve *A* the values of which were obtained by grinding different 1 g. lots of baker's yeast with fine sand in a mortar until a paste was formed, then 4 ml. of 5% (w/v) freshly prepared metaphosphoric acid solution were added and the mixture allowed to stand for varying lengths of time, 5 ml. of water added and the GSH measured in the centrifuged extract. The results are given as mg. GSH/g. of fresh yeast (Fig. 1).

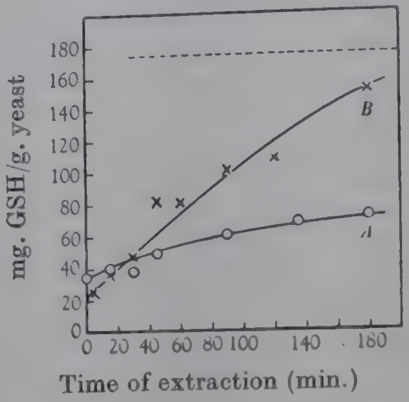

Fig. 1. The extraction of glutathione from yeast by treatment with metaphosphoric and sulphosalicylic acids for various periods. Curve *A*, mg. GSH/g. of fresh yeast obtained by extraction with metaphosphoric acid. Curve *B*, mg. GSH/g. of fresh yeast (another sample) after extraction with sulphosalicylic acid. ----- Total GSH obtained after treatment with ethanol followed by sulphosalicylic acid.

A treatment with sulphosalicylic acid in the same manner yielded a curve *B* of similar appearance, although higher values were obtained. It was observed that a sulphosalicylic acid extract of dried yeast (prepared with ethanol) gave still higher values. The paste from 1 g. of yeast was therefore first treated with 2 ml. ethanol, and then 4 ml. 5% (w/v) sulphosalicylic acid were added followed by 3 ml. water. The amount of GSH found was now further increased and did not change with time. In this manner the amount marked 'total GSH' in Fig. 1 was obtained. Similar results were obtained when metaphosphoric acid was added after ethanol treatment.

The influence of different concentrations of sulphosalicylic acid on the extraction was investigated (Table 1). The same amount of sulphosalicylic acid/g. of yeast was used, and the concentrations of both yeast and sulphosalicylic acid during the extraction were varied simultaneously. After the treatment water was added until the total volume was 10 ml., the final concentration of sulphosalicylic acid being 2%. Even when sulphosalicylic acid was present in the highest concentrations during the denaturation period not all GSH was removed, the total amount found after treatment with ethanol and sulphosalicylic acid being 1·86 mg. GSH/g. yeast.

With trichloroacetic acid, however, a treatment with 2 ml. of a 10% (w/v) solution for 5 min. resulted in total extraction of GSH (1·93 mg. GSH/g. yeast). Addition of ethanol (2 ml.) did not significantly alter this figure (1·85 mg.), while a lower concentration of trichloroacetic acid (4 ml. 5% solution) during the denaturation was quite insufficient, yielding a value (0·50 mg.) almost as low as that (0·20 mg.) obtained by treatment with 2 ml. 10% sulphosalicylic acid alone.

Table 1. *Determination of GSH after treatment of 1 g. yeast with different concentrations of sulphosalicylic acid*

Sulphosalicylic acid solution added			
Initial concentration (%)	Volume used (ml.)	Water added after extraction (ml.)	GSH (mg./g. yeast)
2·5	8	1	0·25
5·0	4	5	0·47
10	2	7	1·18
20	1	8	1·52
40	0·5	8·5	1·55
100	0·2 g.	8·8	1·65

From these results it is seen that both the nature and the concentration of the denaturing agents are of importance for the extraction. The quantitative determination of GSH in yeast may be performed, according to these investigations, either by treatment of 1 g. of yeast with sand and 2 ml. 10% trichloroacetic acid for 5 min., or with sand and 2 ml. ethanol followed by 4 ml. of either sulphosalicylic or metaphosphoric acid in 5% solution. For comparison three 1 g. samples from the same batch of Danish baker's yeast were treated with trichloroacetic acid and with ethanol and sulphosalicylic acid. Trichloroacetic acid treatment gave 2·15, 2·38 and 2·27 mg. of GSH/g. of yeast; while ethanol and sulphosalicylic acid gave 2·25, 2·35 and 2·38 mg. Three types of brewer's yeast were obtained from the Carlsberg brewery: F, U (bottom yeasts) and O (a top yeast). They were washed several times with water and filtered through a sintered-glass filter (G 4) until the dry substance amounted to 25–26%, corresponding to the dry substance in the baker's yeast used. Yeasts F and U contained 1·40 mg. GSH/g., but yeast O only 0·86 mg. The GSH content was thus considerably lower than in the baker's yeast investigated, in which the amount of GSH in the different samples investigated by us usually varied between 1·80 and 2·30 mg. GSH/g.

In order to see whether the denaturing agents also acted differently on cells other than yeast cells we measured the GSH content of rat's liver. The liver, removed immediately after killing the rat, was kept frozen in a freezing mixture. Samples (1 g.) were treated as described for yeast. The following results are typical:

Treatment	mg. GSH/g. liver
Trichloroacetic acid (10%)	1·76, 1·74
Metaphosphoric acid (5%)	1·75, 1·85
Sulphosalicylic acid (5%)	1·74, 1·78
Ethanol and sulphosalicylic acid (5%)	1·80, 1·74

They are concordant and independent of the method, and the differences observed with yeast cells must therefore be due to some peculiarity of these cells. It was of interest to find out whether other constituents of the yeast cell would behave in the same manner as GSH. We compared, therefore, the extraction of GSH with that of total phosphorus. The results are shown in Fig. 2. The mode of release of GSH and of phosphorus from the yeast cells is evidently of a similar nature, and the difficulty of obtaining total extraction is most probably due to the cell membrane. When the yeast was treated directly with 5% sulphosalicylic acid without sand, similar curves were obtained, but the values for the first minutes were considerably lower than with previous treatment with sand. The sand may act solely by destroying the cell membrane, so that the cell contents are more easily released. With water alone only very small amounts of GSH, <0·2 mg./g. yeast, were found; even after 3 hr. extraction without sand no phosphorus was found. Treatment with 2 ml. 10% trichloroacetic acid followed by 7 ml. of water yielded after 10 min. without treatment with sand the total amount of GSH and phosphorus. All these results are best explained by the retaining action of the undestroyed cell membrane.

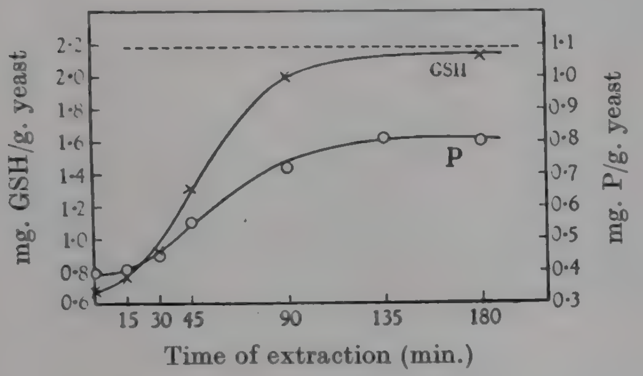

Fig. 2. Comparison of the extraction of GSH and total P from fresh yeast by treatment with 5% sulphosalicylic acid. - - - - Total GSH obtained after treatment with ethanol followed by sulphosalicylic acid.

We therefore tried to destroy the cell membrane with some plasmolyzing agents. 5 g. of yeast were treated for 1 hr. with (*a*) 0·25 ml. chloroform, (*b*) 0·25 ml. of Pirie's (1930) mixture (20 ml. conc. sulphuric acid, 100 ml. ethanol and 80 ml. ether), (*c*) 0·25 ml. of benzene, (*d*) 5 g. NaCl, (*e*) 25 ml. of saturated NaCl solution, (*f*) 25 ml. of a 33% (w/v) solution of urea. With benzene and sodium chloride almost no GSH was extracted (about 0·2 mg./g. yeast). Chloroform and urea were considerably better, yielding about 50% of the total GSH. Pirie's mixture, which released almost all GSH, was best.

Repeated freezing of a sample of yeast acted in the same manner. Eight (1 g.) samples of yeast were placed in centrifuge tubes, frozen in a mixture of ether and solid CO_2, thawed and frozen again, the process being repeated six times in all. The yeast became fluid after the first freezing. Then different denaturing agents were added followed by water until the volume totalled 10 ml. The following amounts of GSH were found:

Treatment	mg. GSH/g. yeast
Sulphosalicylic acid (4 ml. 5%)	2·41, 2·36
Trichloroacetic acid (2 ml. 10%)	2·53, 2·36
Ethanol (2 ml.) and sulphosalicylic acid (4 ml. 5%)	2·33, 2·39
Metaphosphoric acid (4 ml. 5%)	(2·91, 2·95)

In this case all the denaturing agents yielded similar results. Metaphosphoric acid was unsuitable as a slight turbidity appeared on addition of the ammonia solution, resulting in too high values for the GSH content.

DISCUSSION

These results suggest that the difficulties met with in determining substances contained in yeast cells may be caused by the resistance of the cell membrane to the action of the various denaturing agents. However, the large differences observed between the action of agents commonly used for the precipitation and denaturation of proteins were unexpected. While trichloroacetic acid yielded the total amount of GSH, sulphosalicylic and metaphosphoric acids did so only after treatment with ethanol. Our use of ethanol originated from the observation that yeast dried with it yields considerably higher amounts of GSH than fresh yeast. Similar observations had already been made with ethanol (Régnier, 1933), and acetone (Schroeder, Collier & Woodward, 1939), in the isolation of GSH from yeast. Treatment of yeast with ethanol in the analytical determination of GSH has been used previously by Weller (1936) and Bolomey (1939), but in connexion with removal of proteins by means of 10% trichloroacetic acid, where according to our experience it is of no use.

The total GSH determined in yeast after electrolytic reduction was often identical with the amount of reduced GSH present, and in no case did the amount rise by more than 10% after reduction. Our results thus confirm those of Bolomey (1939), and it is our opinion that the large amounts of oxidized GSH found by some authors in yeast may be the result of inaccurate analytical procedures (cf. Fink & Just, 1940).

SUMMARY

1. Different denaturing agents (trichloroacetic acid, sulphosalicylic acid, metaphosphoric acid) release different amounts of GSH and phosphorus from yeast cells.

2. This must be taken into consideration in the estimation of substances contained in yeast cells. Most probably the cell membrane is the cause of the difficulties.

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Preliminary Experiments in the Study of the Respiratory Activity of Micro-organisms Suspended in Thin Films of Fluid Adhering to Solid Surfaces

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It is obvious that the metabolic events taking place in soil occur, under normal field conditions, in the films of water adhering to the soil surfaces. These films vary greatly in extent and thickness, according to soil conditions, e.g. soil structure and moisture content. Evidence secured recently by Lees & Quastel (1946) in their investigations of nitrification in soil show that, even in a soil which is water-saturated but not waterlogged as in their soil perfusion apparatus (Lees & Quastel, 1944), the process of conversion of ammonia into nitrate is brought about by bacteria proliferating at the soil surfaces. It is therefore evident that, for quantitative studies of the biochemical changes taking place in soil, techniques are required which will allow investigations to be made of the metabolism of micro-organisms when these are suspended in films of fluid adhering to solid surfaces, such as those presented by soil.

The well-known Warburg or Barcroft manometric apparatus may be very conveniently adapted for the measurement of the respiration of micro-organisms, when a suspension of these is spread over suitable solid surfaces.

Usually, for estimations of the respiration of micro-organisms, a suspension of the cells in 3 or 4 ml. fluid is placed in the manometer vessel of the Warburg or Barcroft apparatus. The oxygen consumption of the cells, when the apparatus is not shaken, proceeds slowly, the rate being determined by the surface of the fluid in the vessel and the rate of diffusion of oxygen into the fluid. When the apparatus is vigorously shaken new fluid surfaces are constantly exposed to the air in the manometer vessel with the result that the fluid becomes saturated with oxygen. There results an immediate rise in the rate of oxygen consumption by the micro-organisms. The effect of shaking the apparatus is the same as that of spreading the volume of fluid in the vessel over an area so large that the thickness of the film of fluid no longer makes diffusion of oxygen into the fluid a limiting factor. When, therefore, a suspension of the micro-organisms is spread over the surfaces of suitable substances, placed in a vessel attached to a manometer, so that the area of spread is sufficiently great to ensure that diffusion of oxygen into the fluid is not a limiting factor, the resulting rate of oxygen uptake is precisely the same as that obtained when the suspension of cells is agitated in the usual way in the Warburg or Barcroft apparatus.

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EXPERIMENTAL AND RESULTS

We have demonstrated this fact experimentally by placing in a Warburg manometer vessel various substances offering a large surface, over which a suspension of micro-organisms containing excess nutrient is spread, and by showing that the rate of oxygen uptake is the same, when the apparatus is kept quite steady, as when the suspension is placed in a vessel, in the absence of solid adsorbents, and shaken in the normal way.

As examples of solid adsorbents suitable for the spread of suspensions of micro-organisms for the examination of their respiratory activities in the Warburg respirometer we have used filter paper, pumice, Permutit, Decalso, and air-dried soil. As a test organism we have used a 2 % saline suspension of fresh baker's yeast.

Filter paper. A sheet of suitable size Whatman no. 1 filter paper is folded and placed in a Warburg manometer vessel. 1 ml. of a suspension of baker's yeast composed of 0·5 ml. water, 0·2 ml. 10% (w/v) glucose solution and 0·3 ml. of a 2% saline suspension of yeast, is spread drop by drop evenly over the filter paper. 0·3 ml. 20% (w/v) KOH is placed in the inner cylindrical tube of the vessel to absorb CO_2, care being taken that no potash touches the filter paper on which the yeast suspension is spread. In each of two other vessels 1 ml. of the yeast suspension, made up as indicated above, is placed and KOH is put in the inner tubes. No filter paper or other adsorbent is placed in these vessels. All three vessels are attached to Warburg manometers and placed in water baths at 37° and allowed 35 min. to achieve thermal equilibrium. The taps of the vessels are closed after this period. Two of the manometers, one containing the filter paper on which the yeast suspension has been spread and the other containing only the yeast suspension, are not shaken. The third manometer is shaken in the usual way to ensure optimal conditions for aeration of the yeast suspension. The rates of oxygen uptake are recorded in Table 1.

It is necessary, in estimating oxygen uptakes from the manometer readings, to use vessel constants which have been calculated from the expression

$$K_{O_2} = \frac{(V - V_s - V_f)\dfrac{273}{T} + V_f \alpha O_2}{P_0},$$

where

V = total vol. of vessel,

V_s = vol. of solid adsorbent (mass/density),

V_f = vol. of fluid in vessel,

αO_2 = sol. of oxygen in fluid (μl. gas at N.T.P. dissolved in 1 μl. fluid at partial pressure of gas equal to P),

P_0 = normal pressure = $(760 \times 13{\cdot}6)/D$ (expressed in mm. of manometer fluid where D = density of manometer fluid),

T = absolute temperature.

It is assumed that the solubility of oxygen in the solid adsorbent is nil or so small as to be insignificant.

Table 1. *Respiration of yeast; shaken and unshaken, in the presence and absence of filter paper*

	1	2	3
Manometer ...	Static	Static	Shaken
	1 ml. yeast suspension in glucose solution spread over filter paper	1 ml. yeast suspension in glucose solution	1 ml. yeast suspension in glucose solution
Time (min.)	Oxygen uptake (μl.)		
15	65	13	64
30	130	26	139
45	193	38	198
60	265	51	268

It will be seen (Table 1) that under the given experimental conditions the velocity of oxygen uptake by the shaken yeast suspension in presence of the excess glucose, is about five times that of the unshaken yeast suspension. The velocity of oxygen uptake of the yeast suspension spread over the filter paper and not shaken is constant and almost identical with that of the shaken yeast suspension.

These results indicate that the film of fluid in which the yeast is suspended, and which has been spread over the filter paper, is sufficiently thin to allow saturation of the fluid with oxygen under the given experimental conditions.

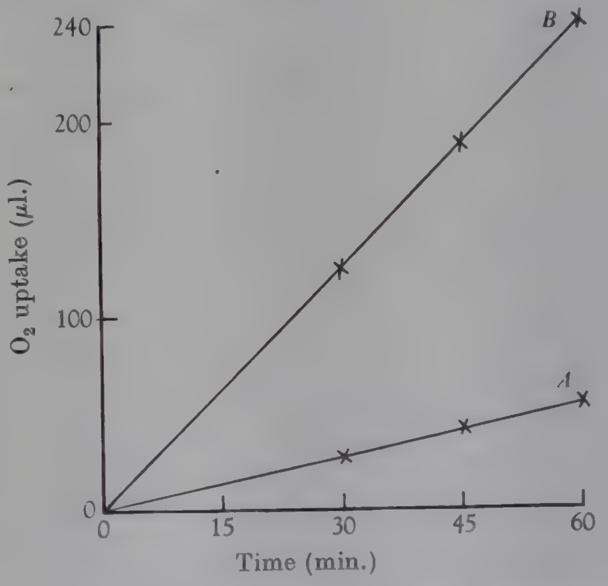

Fig. 1. Rates of oxygen uptake of yeast suspensions (in glucose solution) alone and when spread over pumice. Manometers not shaken. 37°. Air. (*A*) Yeast suspension (in glucose solution) alone. (*B*) Yeast suspension (in glucose solution) spread over pumice.

Pumice. 2 g. of broken pumice (British Drug Houses Ltd. preparation, 10–14 mesh) are placed in a Warburg manometer flask and 1 ml. of the yeast suspension in glucose solution is spread as evenly as possible over the crumbs of the pumice. KOH is placed in the inner tube, and after thermal equilibrium (30–40 min. at 37°) the rate of oxygen uptake is recorded in the usual way, the manometric apparatus, however, being unshaken.

A typical result is given in Fig. 1, which shows also the rate of oxygen uptake of the same volume of yeast suspension not spread over any adsorbent. The rate of oxygen uptake of the yeast suspension spread over the pumice is constant and is identical with that obtained by the suspension when this is shaken in the absence of any solid adsorbent.

These preliminary results would indicate that pumice is a convenient material for the study of the respiration of micro-organisms whose metabolism in films of fluid adhering to a solid surface is the subject of investigation. Air in the manometer vessels may, of course, be replaced by oxygen, if it is thought desirable to test whether the rate of diffusion of oxygen into the pores is a limiting factor.

Permutit and Decalso. The solid adsorbents, Permutit (Permutit Co. Ltd.) (useful as a base exchange material) and Decalso (Permutit Co. Ltd.), may also be used in the Warburg manometric apparatus for the spread of a yeast suspension. Constant rates of oxygen uptake are obtained approximating to that found with a shaken yeast suspension. Results are shown in Table 2.

Table 2. *Respiration of yeast on Decalso and Permutit*

(Warburg manometers static. 37°. Air. Yeast suspension (1 ml. containing 0·5 ml. water, 0·2 ml. 10% glucose solution, 0·3 ml. 2% yeast suspension) placed in a vessel or spread over adsorbents.)

	Manometers not shaken			Manometer shaken
		Decalso	Permutit	
Solid adsorbents...	Nil	(6 g.)	(10 g.)	Nil
O_2 uptake (μl. in 30 min.)	31	148	118	139
O_2 uptake (μl. in 60 min.)	63	292	238	280

Sand. Little or no spread of a yeast suspension takes place over sand, with the result that relatively little increase in the rate of oxygen uptake is obtained in the static manometric apparatus when the cell suspension is distributed over the sand. In one experiment the oxygen uptake in 1 hr. in a static manometer at 37° by 1 ml. yeast suspension (in 2% glucose) spread over Permutit was 240 μl., and by the yeast suspension distributed over 12 g. sand was 99 μl. Thus sand cannot be used as a solid adsorbent for the study of the respiration of yeast, and probably other micro-organisms, suspended in thin films.

Coke. It was thought possible that ground and sieved coke would provide a suitable material for the spread of a suspension of micro-organisms. The specimens of coke we have used have proved to be most unsuitable. When 1 ml. yeast suspension in 2% glucose solution was distributed over 3 g. coke particles, of approximately the same dimensions as the pumice referred to earlier in this paper, the oxygen uptake in 1 hr. at 37° was only 22 μl., less than one tenth of that obtained when the suspension was spread over filter paper. In another experiment, using coke extracted with benzene, acetone and ether, and finally well washed and dried, the oxygen uptake in 1 hr. under the same experimental conditions was 51 μl. Coke, like sand, therefore, may be excluded for the time being from the list of solid adsorbents suitable for the study of respiration of micro-organisms in thin films.

Soil. Air-dried garden soil (4 g.), crumbed and sieved (passing a 2 mm. mesh and retained by a 1 mm. mesh) is placed in each of two Warburg manometer vessels. Yeast suspension (1 ml. composed of 0·5 ml. water, 0·3 ml. 2% yeast suspension in saline and 0·2 ml. 10% glucose solution) is spread drop by drop over the soil crumbs, slowly rotating the manometer vessel to obtain as even a distribution as possible. Glucose solution (1 ml. of a 2%) is spread over the crumbs of the soil in the second manometer flask. KOH is placed in the tubes of the vessels, which are now attached to the manometers and placed in a water bath at 37° to establish thermal equilibrium. After 30–40 min. the taps of the vessels are closed and the rates of oxygen uptake are recorded, the manometers not being shaken. Constant rates of oxygen uptake are obtained and these are recorded in Fig. 2. The rate of oxygen uptake of the 1 ml. yeast suspension in 2% glucose solution spread over 2 g. pumice is also shown in Fig. 2.

There is a fairly considerable oxygen consumption by the soil over which glucose solution alone has been spread, this obviously being due to the micro-organisms present in the soil. The rate of oxygen uptake, secured by the yeast suspension in glucose solution spread over the soil, is much greater than that obtained when the glucose solution alone is added to the soil, and is appreciably greater than that obtained by the yeast suspension in glucose solution spread over the pumice. Examination of the figures recorded in Fig. 2 shows that the difference between the rate of oxygen consumption, in soil, of the yeast suspension in glucose solution, and of that of the glucose solution alone spread over the soil, is almost identical with that obtained by the yeast suspension in glucose solution spread over the pumice. This conclusion would be expected if the yeast suspension spreads as a thin film over the soil crumbs, as has been shown in the case of pumice, and if the oxygen uptake of the yeast in the film of fluid adhering to the soil crumbs is unaffected by the respiration of the soil's own microflora. Other experiments, similar to that whose results are recorded in Fig. 2, have been carried out with yeast suspensions and yield similar results. Since there is no reason at present to believe that in an experiment of short duration the respiration of yeast, in presence of excess of nutrient (glucose) and free access of oxygen, would be affected by that of the soil's microflora, it follows that soil crumbs may conveniently be used, like those of pumice, for the study of the respiration of micro-organisms in thin films.

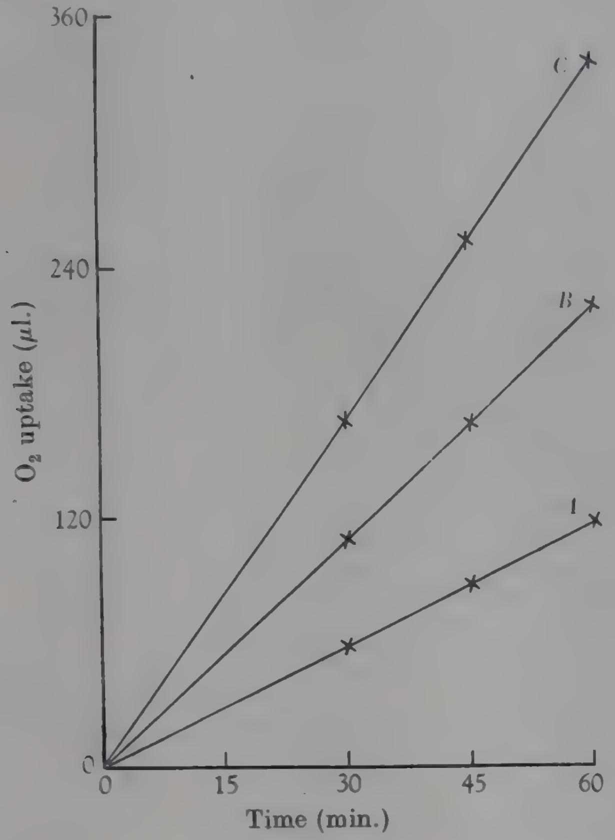

Fig. 2. Rates of oxygen uptake of yeast suspensions (in glucose solution) spread over air-dried soil crumbs and pumice, and of glucose solution spread over soil. Manometers not shaken. 37°. Air. (*A*) 1 ml. 2% glucose solution spread over 4 g. air-dried garden soil. (*B*) 1 ml. yeast suspension (in 2% glucose solution) spread over 2 g. granular pumice. (*C*) 1 ml. yeast suspension (in 2% glucose solution) spread over 4 g. air-dried garden soil.

The fact that the soil has a highly mixed microflora with a variable respiratory activity, depending on the availability of organic substrates, oxygen and water, is of course a complicating factor when studies are being made of the respiration of suspensions of micro-organisms added to soil crumbs. Use, however, may be made of the fact that many micro-organisms have very high respiratory activities, and small quantities of such organisms may secure rates of oxygen uptake far greater than that obtained by the soil alone, on which the suspensions of the organisms are spread.

The adsorbent and the nutrient medium. If an adsorbent alters the constitution of a nutrient medium, by removal of one or more constituents, an effect on the respiration of the micro-organisms under investigation might be expected to take place. So far, in our preliminary experiments, we have not noticed effects of this character. It is clear, however, that our experimental technique should make it possible to explore such effects of adsorbents on the respiratory metabolism of micro-organisms.

Effect of water addition to the solid adsorbents

It is obvious that, as the water content of the adsorbents increases, so will the films of fluid on the adsorbent surfaces increase in thickness. If micro-organisms are suspended in these films, the rate of oxygen uptake of the cells will not be affected by the changed water content until the films assume a thickness where diffusion of oxygen into the fluid becomes a limiting factor. The velocity of oxygen uptake will then fall, the rate of fall being dependent on the rate of waterlogging of the adsorbent. Studies, therefore, of the rates of oxygen uptake of micro-organisms suspended in various volumes of fluid added to a constant quantity of a solid adsorbent will indicate the point at which waterlogging occurs, for at this point there will be a sudden drop in the rate of oxygen consumption. This point, at which waterlogging occurs, is clearly a measure of the water-holding power of the adsorbent; the greater its water-holding power, the greater the amount of fluid which can be added to the adsorbent before waterlogging occurs.

Consequently a greater volume of fluid containing a standard quantity of micro-organisms may be added to an adsorbent of high water-holding power, before a drop in respiration takes place, than can be added to an adsorbent of lesser water-holding power before a similar drop in respiration occurs.

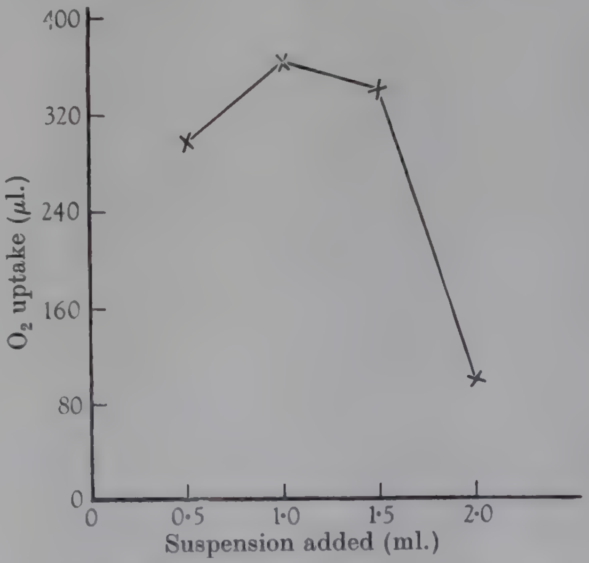

Fig. 3. Variations of oxygen uptakes of yeast suspensions (in presence of glucose) in varying volumes of fluid spread over air-dried soil. Abscissa: Vol. fluid (containing 0·3 ml. 2% yeast suspension and 0·2 ml. 10% glucose) added to 4 g. air-dried allotment soil. Manometers static.

Typical results showing the effects of increased moisture content of soil on the respiration of an added suspension of yeast are shown in Fig. 3.

Respiration is greatest at a moisture content corresponding to the addition of 1 ml. fluid to 4 g. air-dried soil crumbs, i.e. to a water content of 20 % (wet soil). Under the given experimental conditions, greater moisture contents than this lead to the filling up of soil pores with resultant fall in respiration as access of oxygen to the yeast cells becomes more and more restricted. It is noteworthy that under the conditions employed in the experiment, whose results are given in Fig. 3, the highest rate of respiration of the yeast in presence of the soil was 367 μl. O_2 uptake in 1 hr.; that of the soil in presence of glucose solution (1 ml. fluid to 4 g. air-dried soil) was 91 μl. The difference between these two figures is 276 μl. which would be expected to be the oxygen uptake in 1 hr. of the added yeast suspension independent of that of the soil's own microflora. The actual oxygen uptake of the yeast suspension under optimal aeration conditions (shaken Warburg apparatus in absence of soil) was found to be 253 μl. in 1 hr. Our colleague Dr D. M. Webley has investigated the rates of respiration of standard quantities of bacteria of high respiratory activities, suspended in various volumes of fluid, when these are spread over soils (Webley, 1947). Using this technique he has shown how bacterial suspensions may be used for studying problems of aeration into soils. Quastel & Webley (1947) have used this manometric method of assessment of soil aeration for estimating the effects of incorporation with soil of salts of alginic acid and of other organic materials on soil air-water relationships. The addition of sodium alginate to a soil increases its water-holding power; this is shown by the fact that bacterial suspensions spread over soils containing alginate respire at much greater rates at high water contents, than when they are spread over soils containing the same amount of water but with no added alginate. The alginate does not serve as a nutrient source or stimulant to the bacteria, as shown by control experiments; its effect is to afford increased oxygen access to the soil crumbs at the high water contents. The effects of a variety of other substances, e.g. straw, farmyard manure, sawdust, cellulose derivatives, which are all of agricultural importance as organic fertilisers, on the water-holding power of soils may be compared in this way.

SUMMARY

1. If a small volume of a yeast suspension in glucose solution is spread over a suitable solid adsorbent, placed in a Warburg or Barcroft manometer apparatus which is kept unshaken throughout the experiment, its rate of respiration is constant, and is identical with that of the yeast suspension in glucose solution when this is shaken in the normal way in the absence of solid adsorbents.

2. Suitable materials for the spread of suspensions of yeast, and presumably other micro-organisms, so that their respiratory activities in thin films of fluid can be examined, are pumice, Permutit and Decalso. Filter paper also offers an excellent medium for the spread of a suspension of organisms for the study of their respiratory activities.

3. The rate of respiration of a small volume of yeast suspension in glucose solution spread over soil crumbs is constant and greater than that obtained when the suspension is spread over pumice or filter paper. If the rate of respiration due to the soil's own microflora in presence of glucose is subtracted from the rate of respiration of the yeast suspension in presence of glucose spread over the soil, a value is found which is identical with that obtained by the yeast suspension spread over pumice.

It is concluded that soil crumbs, like pumice, form a suitable medium for the spread, as thin films, of small volumes of suspensions of micro-organisms. Soil crumbs may therefore be used for investigations of respiratory activities of micro-organisms suspended in thin films of fluid.

4. Sand and coke are unsuitable materials for the spread of yeast suspensions.

5. The effects of altering the water content of a solid adsorbent on the respiratory activity of micro-organisms spread over the adsorbent are pointed out. A means is indicated of utilizing measures of rates of respiration of organisms suspended in varying volumes of fluid and spread over a standard quantity of solid adsorbent for the study of the water-holding power of the adsorbent, and the aeration conditions within it at various moisture contents. The applicability of this to soil is discussed.

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Further Tests on Human Enamel Protein

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In previous work (Pincus, 1936, 1939) it was shown that human enamel protein differs from the keratins amongst which it had been classed (Rosebury, 1930). It contained 12·1 % N, 1·2 % S, while cystine was either absent or present in low concentration; tyrosine was also present. Enamel protein resisted digestion both by pepsin and trypsin, and was unaffected by KCN, Na_2S, and sodium thiolacetate. This limited examination has now been extended.

METHODS

Preparation of material. Unerupted human molar teeth were used for experiments throughout; these buried teeth were removed surgically from patients aged from 18 years upwards, and kept at room temperature in water saturated with chloroform. The growth of moulds and bacteria has been avoided in the present experiments by using the protein within a few days after extraction of teeth.

The teeth were brushed freely with 0·5 % Na_2CO_3 solution and with water, the roots separated from the crowns and discarded. Protein was separated from enamel by steeping in 0·5 M-HCl for 24 hr. at room temperature. Dentine was then discarded, and the tiny collections of protein washed in water. Tests have all been carried out on specimens about 3 or 4 mm. diameter at their largest and probably less than 0·1 mm. thick. Prior to 1939, microscopic examination had suggested that the contents of grooves on the occlusal (biting) surface of molar teeth sometimes exceeded the mere surface layer known as Nasmyth's membrane (Nasmyth, 1839). This appearance was thought to be due to debris of section cutting. Table 1 shows results at this stage of the work. However, the existence of two parts in enamel protein has now been recognized, and separate examination of them has been carried out. After the exposure of the teeth to acid (as above), the protein sometimes falls away from the enamel, lying loose. When this occurs, Nasmyth's membrane is seen as a diaphanous structure, while the groove protein is more nearly a tiny solid mass, sometimes of yellowish tinge. Separation was carried out visually under water by the use of a smooth-ended glass pipette 6–8 cm. long, and about 7 mm. diameter. The original name, Nasmyth's membrane, has been retained for the surface layer, i.e. the protein adjacent to the enamel surface; the other part is termed groove protein. Fig. 1 shows the relation of these structures to each other and to the tooth.

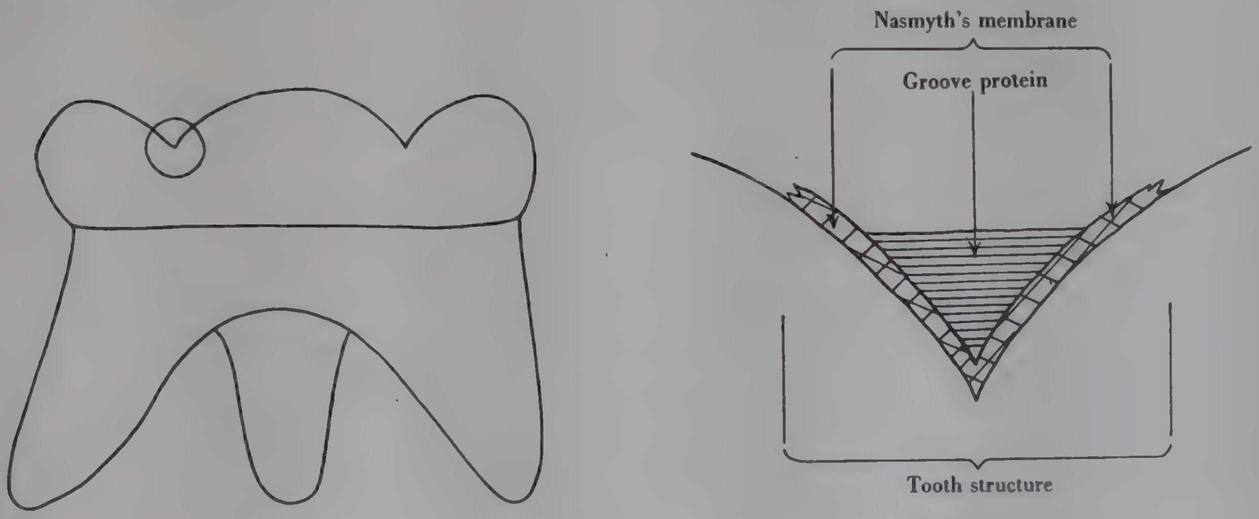

Fig. 1*a*. Fig. 1*b*.

Fig. 1 (*a*). Diagram of molar tooth. Position of occlusal groove is marked by a circle. (*b*) Diagrammatic enlargement of circle in (*a*); the positions occupied by Nasmyth's membrane and groove protein in the occlusal groove are indicated.

RESULTS

Three N estimations shown in Table 1, carried out on mixed Nasmyth's membrane and groove protein, average 11·8 %. The low N and the presence of carbohydrate (see Table 2), provide some evidence as to the biochemical nature of these structures; in considering the estimations, difficulties met in separating tooth proteins from Ca salts must be borne in mind. The N estimations may be compared with those given by Levene (1925) for mucoproteins, where values range from 6·7 % in oviduct (Giacosa, 1882) to 13·3 % in submaxillary glands (Hammarsten, 1888).

Table 1. *Analysis of Nasmyth's membrane mixed with groove protein*

(*a*) Quantitative tests (as %, corrected for ash)

	(Schoeller)	(Weiler)
N	12·1	11·1, 12·1
S	1·2	—

(*b*) Qualitative tests (hydrolysis at 108°, 30 min., pH 8·0)

Xanthoproteic	+
Biuret	– or faint

Table 2 shows that differences of a biochemical nature exist between the two proteins in occlusal grooves. Cupriethylenediamine (CuEn) stains Nasmyth's membrane black, and there is some reason to believe that a Cu protein complex is formed. Some CuEn protein complexes have been examined, and have been found to be bacteriostatic and possibly bactericidal. The groove protein dissolves immediately in CuEn. The purple colour seen on acid hydrolysis of groove protein is absent when Nasmyth's membrane is subjected to acid hydrolysis; the Molisch test is positive with groove protein and negative with Nasmyth's membrane. Cupriammonium solutions dissolve groove protein immediately while Nasmyth's membrane resists for days and is stained black. Since the Molisch test shows the presence of carbohydrate, the possible origin of this carbohydrate is of interest. Glycogen was shown by Glock (1940) to be very abundant in mouth epithelium and in other structures adjacent to the developing molar teeth of foetal and very young rats. Examination for the presence of pentose (in the present work) proved negative. Symington & Rankin (1908) have shown X-ray pictures in which human molar teeth may be seen calcifying downwards from the tip of each cusp, the sides eventually meeting to form a groove. Coalescence of cusps to form a complete occlusal surface occurs normally, and in such a process, developmental material may be 'trapped' in the groove.

While relatively few suitable teeth are available, a rigid selection of material ensures that films of mucin or other adventitious material are kept at a minimum. It is obvious that the tenuous layer on the surface of newly formed enamel, Nasmyth's membrane, is soon removed from exposed surfaces by wear, while in secluded positions such as narrow fissures on the occlusal surface, it may persist. This persistence may also apply to such other contents of fissures as are described above, and may lend to the present work an added value. Such narrow fissures are a very common site of dental caries.

Table 2 shows that exposure to Schweitzer's solution results in histological differences in the structure of groove protein becoming apparent. One part disappears promptly; this is thought to be the remnant 'caught up' as it were during the formation of the tooth, in the groove between the cusps, and

Table 2. *Qualitative tests on Nasmyth's membrane and groove protein separately*

	Nasmyth's membrane	Groove protein
Conc. HCl at 37° for 24 hr.	Dissolves	Dissolves; purple colour
Conc. HCl; boiling water bath	Dissolves >30 min.	Dissolves <2 min.; purple colour
Millon	+	+
Ninhydrin	–	Not tested
Pepsin at 37°	Very slow attack	Very slow attack
Trypsin at 37°	Very slow attack	Very slow attack
Gram – bacillus at 37° (see note *a*)	No attack seen in 14 days	Not tested
Lactobacillus acidophilus at 37°	Not tested	No attack seen in 14 days
Schweitzer's solution at room temperature	Resists	The greater part dissolves; a small part resists (see note *b*)
Cupriethylenediamine at room temperature	Resists for days and is stained black	Dissolves in few seconds
Thioglycollic acid at room temperature	Resists 24 hr.	Dissolves in about 1 min.
Acetic acid	Swells	Not tested
Formol (10%)	Toughened	Toughened
Molisch	–	+
Aniline acetate	–	–
Phloroglucinol	Not tested	Red colour; ppt. No pentose detected spectroscopically

(*a*) This Gram – bacillus was isolated from dental carious lesions (Pincus, 1948), and its proteolytic action on some proteins (e.g. serum, sericin) at 37° has been studied. The rise in free amino groups which is noted during the first week or so of digestion is sometimes followed by a decrease, due to subsequent bacterial degradation. Estimations were thus made over the period where the titre of free amino groups was rising, i.e. where the proteolytic power of the organism could be demonstrated.

(*b*) Microscopical examination showed markings resembling enamel prisms, with a few epithelial cells.

persisting there. This remnant is of mesodermal origin. That part of groove protein, which resists exposure to Schweitzer's solution, is probably a fragment of Nasmyth's membrane which existed in histological continuity with the groove protein, and was inadequately separated from it by the use of a pipette, and the gentle suction used.

In its resistance to chemical and bacterial attack, and to pepsin and trypsin, some resemblance between Nasmyth's membrane and silk fibroin had been pointed out to the writer by the late Sir Patrick Laidlaw prior to 1939. Later, the work of Takamatsu (1933) came to notice, describing the solution of silk in cupriethylenediamine, after which a test of the effect of this substance on enamel proteins seemed to be of interest. Clinical trial of local application of cupriammonium solutions for caries prevention is contemplated, when a suitable technique has been evolved.

SUMMARY

1. Human dental enamel protein has been shown to consist of two proteins, Nasmyth's membrane and groove protein; results of some tests on these are given.

2. Both contain tyrosine, and both resist enzymic attack. Groove protein contains carbohydrate, possibly glucose. In cupriammonium solutions the two structures are sharply differentiated, Nasmyth's membrane resisting solution and staining black, while groove protein readily dissolves.

The writer wishes to thank Professor Margaret Murray for help, Dr Howitt for supplies of cupriethylenediamine and Mr Taylor for the supply of clinical material. To the Westminster Hospital Medical School, which has kindly provided facilities, and the Medical Research Council, which has provided grants, the grateful thanks of the writer are recorded.

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Properties of Glucose Oxidase (Notatin)

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A glucose oxidase was discovered by Müller (1928, 1936) in *Aspergillus niger* and *Penicillium glaucum*. He found that this enzyme catalyzes the oxidation of glucose to gluconic acid by means of molecular oxygen, and that at a much slower rate it also oxidizes mannose and galactose but not other sugars. Since the activity of this enzyme was found by him not to be affected by cyanide he concluded that it does not depend upon the presence of an active heavy metal.

The enzyme was reinvestigated by Franke & Lorenz (1937) and by Franke & Deffner (1939) who, working with a more purified preparation, demonstrated that during the catalytic oxidation of glucose to gluconic acid, O_2 is reduced to H_2O_2. They found also a certain proportionality between the activity of different preparations and their flavine content and concluded that the enzyme must be a flavoprotein. Although their enzyme preparation was 80 times more active than that of Müller, we now realize that it was not more than 10 % pure. Unlike Müller they found that in addition to O_2 other substances such as indophenols may also act as hydrogen acceptors although their efficiency is much lower.

Müller (1941), from a study of the oxidation of various sugars by his enzyme preparations both in O_2 and anaerobically using 2:6-dichlorophenolindophenol, concluded that two enzymes were present, one reacting directly with O_2 and a second which reacts only with certain other H acceptors. Franke (1944) has criticized this conclusion of Müller and also those of Ogura (1939) who claimed to have isolated from *Aspergillus oryzae* a glucose-oxidizing enzyme which could not utilize O_2 as H acceptor. According to Franke (1944) the glucose oxidases from *Penicillium glaucum*, *Aspergillus niger* and *A. oryzae* are virtually identical and consist of a single aerobic oxidase. The findings of Müller (1941) and Ogura (1939) may have resulted from the low purity of their preparations; their Q_{O_2} values being of the order of 100 at 25° in air, whereas Franke was able to prepare a much purer material ($Q_{O_2} = 8000$ at 30° in O_2). Moreover, the highly purified oxidase ($Q_{O_2} = 130,000$) that we have used for the experiments described in this paper can utilize both O_2 and 2:6-dichlorophenolindophenol as H acceptors.

The almost complete purification of glucose oxidase from culture media of *Penicillium notatum* was achieved by Coulthard, Michaelis, Short, Sykes, Skrimshire, Standfast, Birkinshaw & Raistrick, (1942, 1945). They demonstrated that the antibiotic 'notatin', which is thermolabile and only effective in presence of glucose and O_2, is identical with the glucose oxidase of Müller and that its bactericidal action is due to H_2O_2 produced during the oxidation of glucose: $C_6H_{12}O_6 + O_2 + H_2O = C_6H_{12}O_7 + H_2O_2$. The same conclusion was arrived at independently by van Bruggen, Reithel, Cain, Katzman, Doisy, Muir, Roberts, Gaby, Homan & Jones (1943) who purified this enzyme and described it under the name Penicillin B. Their experiments suggested that the prosthetic group of the enzyme was the alloxazine-adenine dinucleotide. Working with the highly purified enzyme we were able to demonstrate that this dinucleotide is indeed the prosthetic group of notatin (Keilin & Hartree, 1946).

Glucose oxidase, notatin and penicillin B therefore designate the same enzyme which catalyzes the aerobic oxidation of glucose to gluconic acid. As a matter of convenience we shall use in our descriptions the short term notatin.

The first part of this paper will deal with the constitution and physical properties of notatin while the second part will consist of a general account of the kinetics of the pure enzyme.

EXPERIMENTAL

A. *Physical properties and constitution of the enzyme*

Notatin* was obtained as a pale yellow powder which dissolved freely in water to give a bright yellow solution with a faint opalescence. On centrifuging, a perfectly clear solution was obtained plus a small purplish residue constituting < 10 % of the preparation.

Absorption spectrum and equivalent weight. A solution of notatin in water containing 10·0 mg./ml. was centrifuged to remove insoluble impurities. It then contained 9·12 mg./ml. This solution was diluted with half its volume of 0·1 M-phosphate buffer pH 6·5 and the absorption curve determined in a 1 cm. layer (Curve I, Fig. 1) using a Beckman photoelectric quartz spectrophotometer. A typical flavine type of absorption spectrum was obtained with maxima at 377 and 455 mμ. van Bruggen *et al.* give the positions of these maxima as 375 and 452 mμ. Addition of glucose to the solution in a closed vessel gives rise to the autoxidizable dihydro or leuco form of the enzyme which is practically colourless (curve II). A more complete reduction to the leuco form was obtained in presence of $Na_2S_2O_4$ (curve III) though the strong absorption by this salt in the ultraviolet region precludes any determination of absorption below 400 mμ.

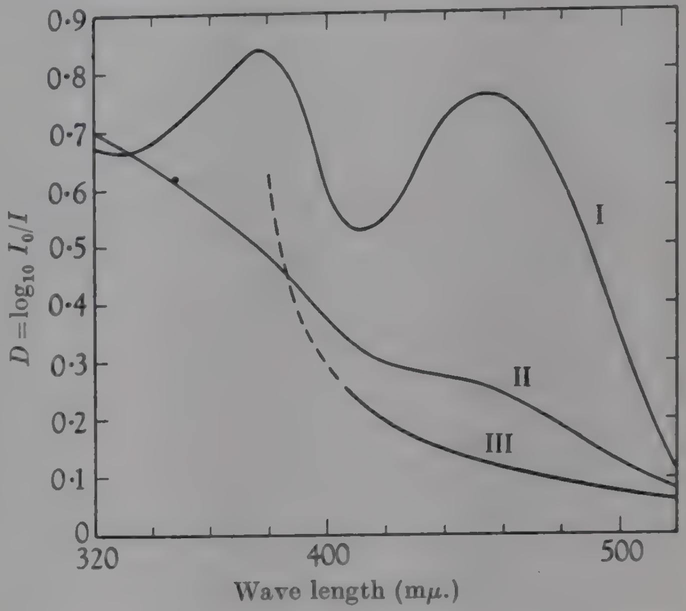

Fig. 1. Absorption spectra of a 0·608% solution of notatin in 0·03 M-phosphate buffer pH 6·5. I, without reducing agent. II, +glucose. III, $+Na_2S_2O_4$.

The absolute value of the height of the absorption band near 450 mμ. is fairly constant for all flavoproteins and the average value of the absorption constant is $\beta = 2{\cdot}4 \times 10^7$ (Straub, 1939). From this figure it is possible to estimate the minimum molecular weight of the enzyme; i.e. the weight of enzyme containing 1 mol. of the alloxazine-adenine dinucleotide. Thus $\beta = (2{\cdot}303\ DM)/cl$, where D = optical density (0·76) = $\log_{10}$ incident light/transmitted light, M = molecular weight, c = concentration in g./ml. (0·00608), and l = optical depth in cm. The calculated value of M is 83,000 assuming the enzyme to be completely pure. The correction for colourless impurities will lower this figure while flavoprotein impurities will probably not change it. From the experiments described below it appears that the flavine-free impurities do not exceed 10 %, hence the equivalent weight is about 75,000 and the molecular weight a simple multiple of this figure. As the molecular weight of this enzyme determined by Ogston & Cecil (see addendum to this paper) was found to be about 152,000, each molecule of enzyme contains two prosthetic groups.

Coulthard *et al.* determined the absorption spectrum of a similar highly purified preparation and deduced a purity of 90 % from the absorption at 450 mμ. Whereas their value of the height of this band agrees very closely with ours, they obtained a considerably higher figure for the second band near 380 mμ.

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* The notatin used in these experiments was kindly supplied by Dr W. F. Short (Coulthard *et al.* 1945).

Electrophoresis. Fig. 2 summarizes the results obtained in the Tiselius electrophoresis apparatus. The material is seen to be almost homogeneous. A small fraction migrates more rapidly than the oxidase and a trace of a more slowly moving fraction can just be detected. Further evidence on the homogeneity of the oxidase was obtained from its behaviour in the ultracentrifuge (see addendum to this paper).

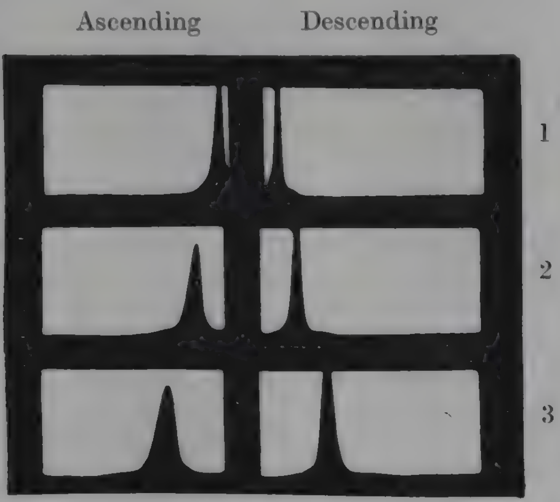

Fig. 2. Migration of glucose oxidase in the Tiselius electrophoresis apparatus. 1·67 % solution. 0·05 M-phosphate buffer pH 6·42. Current 19 milliamp. at 250 V. Temp. = 1·5°.

Exposure no.	Time (min.)	Schlieren angle
1	28	65°
2	49	65°
3	80	55°

Nature of the prosthetic group. From the work of van Bruggen *et al.* (1943) it can be concluded that the prosthetic group is probably the alloxazine-adenine dinucleotide (A.A.D.) common to diaphorase, D-amino-acid oxidase and most other flavoproteins. It has not so far been possible to split off the prosthetic group of glucose oxidase and at the same time obtain the protein in a native state. Were it possible to obtain the native protein, then a restoration of catalytic activity on addition of A.A.D. would identify the latter as the prosthetic group. The alternative method, as was suggested by Coulthard *et al.* (1945), is to add the prosthetic group of notatin to the native protein of D-amino-acid oxidase. The prosthetic group of D-amino-acid oxidase is known to be A.A.D. (Warburg & Christian, 1938), hence, as the addition of boiled notatin to the amino-acid oxidase protein restores the specific catalytic activity of the latter (Keilin & Hartree, 1946) the two enzymes must have a common prosthetic group.

The D-amino-acid oxidase protein was separated from its prosthetic group according to the method of Negelein & Brömel (1939) and the enzyme was then reconstructed from 5 mg. free protein (fraction B of Negelein & Brömel) and varying volumes of a standard solution of A.A.D. containing 0·65 mg. riboflavin phosphate/ml. The catalytic activities of the mixtures were estimated manometrically by the rates of oxidation of DL-alanine in pyrophosphate buffer pH 8·3 at 39° in presence of catalase. The relationship between the volume of A.A.D. solution added and the catalytic activity of the system is shown in Fig. 3. The activity of the amino-acid oxidase protein was now tested under the same conditions with the prosthetic group of notatin in place of the A.A.D. solution. For this purpose a solution of notatin in water containing (after centrifuging) 2·77 mg./ml. was heated at 100° for 15 min. and cooled. The addition of 0·02 and 0·04 ml. of this solution to the above oxidizing system brought the amino-acid oxidase activity to the same level as would the additions of 0·00049 and 0·00102 ml. respectively of the standard A.A.D. solution (Fig. 3).

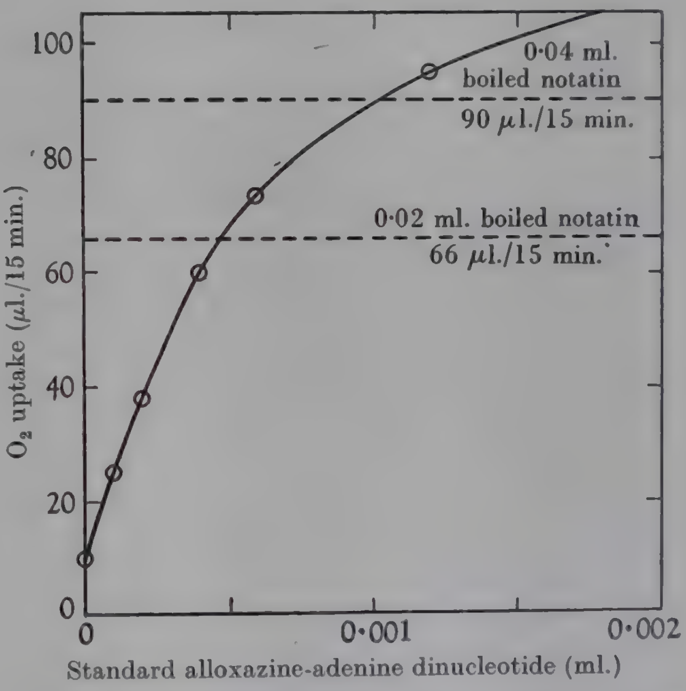

Fig. 3. Rates of O$_2$ uptake obtained on adding various amounts of alloxazine-adenine dinucleotide or of boiled notatin to the system: 5 mg. D-amino-acid oxidase protein, 12 mg. DL-alanine, 0·1 ml. catalase, 0·07 M-pyrophosphate buffer pH 8·3 to 3·3 ml. Temp. = 39°. Standard dinucleotide solution for calibration (circles) contained 0·65 mg. riboflavin phosphate/ml.

Hence 1 ml. boiled notatin solution containing 2·77 mg. notatin is equivalent to 0·025 ml. of the standard A.A.D. or to 0·0163 mg. riboflavin phosphate. The sample of notatin therefore contains 0·59 % riboflavin phosphate. Pure diaphorase contains 0·66 % riboflavin phosphate (Straub, 1939), hence if we assume that the riboflavin contents of diaphorase and notatin are the same, and that all the flavine in the preparation is present in the form of the enzyme, then the notatin is about 90 % pure. On the other hand, if the notatin contains flavoprotein impurities this estimate is too high. Thus Ogston & Cecil (addendum to this paper) estimate the purity to be 80 % or possibly more, whereas the agreement (above) between their value of the molecular weight and our estimate of the equivalent weight, based on the assumption that there are no flavoprotein impurities, is very close. The purity of the notatin is therefore between 80 and 90 %.

Effect of temperature and pH *on the fluorescence of notatin.* Pure notatin does not fluoresce in ultraviolet light although the strong green fluorescence of A.A.D. appears when the enzyme is denatured. In this respect it differs from diaphorase which fluoresces both before and after denaturation. At temperatures below 60° no fluorescence can be detected while at 70° it develops very rapidly. Experiments were carried out at intermediate temperatures in an attempt to reverse the phenomenon by sudden cooling. In no case was there a decrease in the fluorescence produced by the higher temperature.

The effect of pH on the fluorescence is parallel to its effect on enzyme activity (see below). The solution becomes fluorescent at pH >8 or <2, i.e. outside the range of catalytic activity. Here again there was no evidence of reversibility of the appearance of fluorescence or of enzyme activity on rapid neutralization to pH 5. Coulthard *et al.* (1945) showed that the bactericidal properties of notatin rapidly disappear outside the pH range 2–8.

B. *Kinetics of the enzyme reaction*

It was shown by previous workers that oxidation of glucose to gluconic acid catalyzed by glucose oxidase is accompanied by reduction of O_2 to H_2O_2 (p. 221). The formation of H_2O_2 in this primary oxidation reaction has been confirmed by the addition of azide-catalase which rapidly turns from greenish brown to red and changes the pattern of its absorption spectrum (Keilin & Hartree, 1945*a*). It can also be demonstrated by the utilization of this peroxide in a secondary or coupled oxidation of alcohols catalyzed by catalase (Keilin & Hartree, 1945*b*).

Notatin was made up as a 0·1–0·2 % aqueous solution, which was stable for 1 week at 5°, and suitable dilutions were prepared as required. Measurements of O_2 uptake were carried out in air in Barcroft differential manometers at 39° (unless otherwise stated). As a general rule the right-hand flasks of the manometers received 0·3 ml. notatin, 0·3 ml. glucose, 0·1 ml. catalase (Keilin & Hartree, 1945*a*) and buffer to 3·3 ml. The left-hand flasks received 3·3 ml. buffer.

Influence of pH *and of buffer concentrations.* The O_2 uptake of the system composed of 15 μg. notatin, 0·3 ml. 10 % glucose and 0·1 ml. catalase was determined in various buffers over the pH range 2·3–10·3 (Fig. 4). The buffer concentrations were about 0·2 M and the pH's of the reaction mixtures were determined with a glass electrode. The optimum pH is 5·6 which is in close agreement with the figure given by Müller (1936) for the enzyme from *Aspergillus niger*. Variations of phosphate and acetate buffer concentrations between 0·03 and 0·25 M do not influence the rates of O_2 uptake. The loss of activity above pH 8 is noteworthy and the effect of high pH was further investigated as follows:

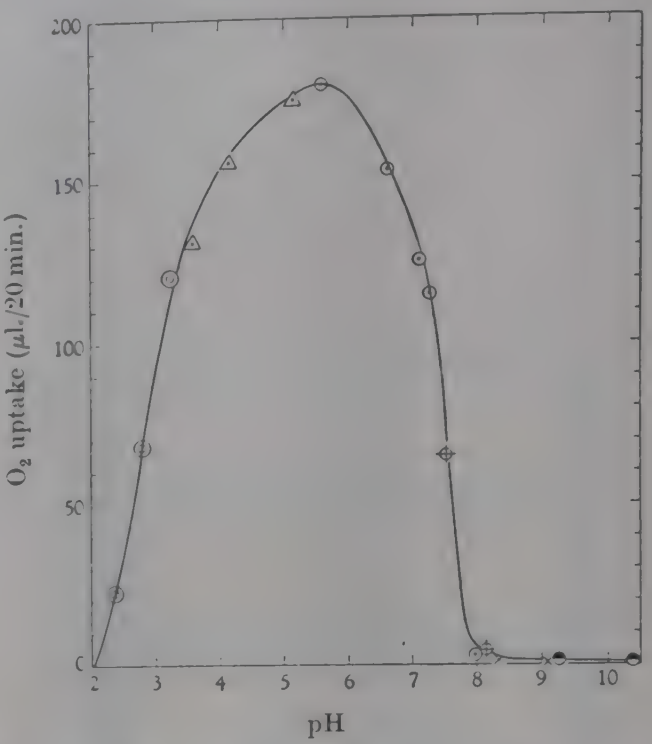

Fig. 4. Effect of pH on the activity of glucose oxidase. 15 μg. notatin, 1 % glucose, 0·1 ml. catalase. 0·2 M-buffers, Temp. = 39°. ◎ phosphate-citrate; ⊕ phosphate borate; △ acetate; × pyrophosphate; ⊙ phosphate; ◓ phosphate-NaOH.

Three borate buffers were prepared as shown in Table 1. Notatin was incubated for various periods in these buffers, and then brought to pH 5·3 by addition of the previously determined volume of 0·2 M-acetate buffer pH 3·6. The activity of the enzyme was then determined under the standard conditions with glucose and catalase at pH 5·3. Table 1 gives the volumes of acetate buffer required to neutralize the borate buffers to pH 5·3. Thus in one set of experiments an aqueous solution of notatin (1·25 mg./ml.) was diluted 50 times in the mixture borate I + acetate (Table 1) and 0·5 ml. tested in presence of catalase and 1 % glucose at 39°; the volume being made up to 3·3 ml. with the mixed buffer (Exp. 1, Table 2). A second sample of the aqueous notatin was similarly diluted with borate I alone. At intervals up to 40 min. 0·5 ml. was pipetted into a Barcroft flask containing 1·5 ml. borate I + 1·0 ml. acetate buffer. The enzyme was then tested with glucose and catalase as before (Exps. 2–5, Table 2).

Table 1. *Acidities of borate buffers used to test destruction of notatin at high* pH

Buffer	Constitution	pH	Volume of 0·2 M-acetate buffer pH 3·6 added to 10 ml. of the borate buffer to give final pH 5·3 (±0·1) (ml.)
Borate I	0·05 M-borax	9·1	5
Borate II	100 ml. I + 50 ml. 0·1 M-KH_2PO_4	8·1	3
Borate III	100 ml. I + 75 ml. 0·1 M-KH_2PO_4	7·6	2·5

Table 2. *Destruction of notatin by incubation at room temperature at* pH 9·1

(12·5 μg. notatin, 1% glucose, 0·1 ml. catalase.)

Exp. no.	Time of incubation at pH 9·1	Activity after neutralization to pH 5·3 (μl. O_2/25 min.)
1	0	296
2	2	25
3	7	24
4	23	7
5	40	0

Table 2 shows the rapid disappearance of enzyme activity that follows incubation at the high pH. These results are plotted in Fig. 5 (line *A*) together with similar experiments in which the incubation pH was 8·1 and 7·6. Experiments in which glucose was present during the incubation period (dashed lines) show that the substrate can protect the enzyme to a considerable extent from destruction at pH > 8.

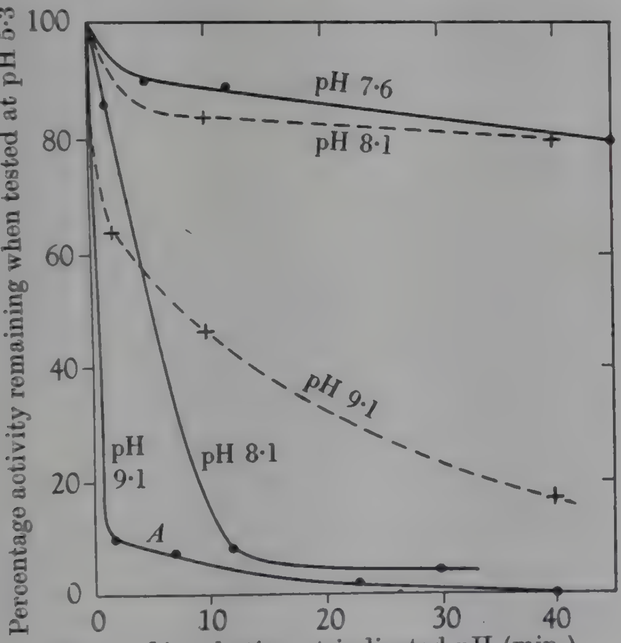

Fig. 5. Loss of activity of glucose oxidase following incubation at room temperature at pH 7·6, 8·1 and 9·1 for various periods prior to neutralization to pH 5·3 and testing with glucose at 39°. 12·5 μg. notatin, 1% glucose, 0·1 ml. catalase. ●—● incubation in absence of glucose. ×—× incubation in presence of glucose.

Unless otherwise stated, all subsequent experiments were carried out in 0·2 M-phosphate buffer pH 5·6.

Effect of substrate concentration and oxygen tension at 39°. The effect of glucose concentrations was determined with 5 μg. notatin and glucose concentrations up to 7·3%. From the results obtained (Fig. 6) the Michaelis constant is found to be $K_m = 0{\cdot}0042$ M which is considerably lower than the value 0·026 found by Franke & Lorenz (1937) with a crude enzyme preparation.

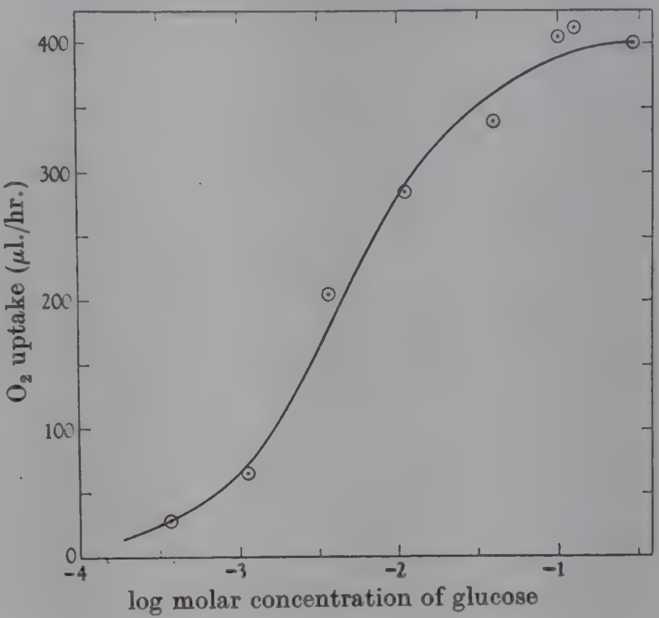

Fig. 6. Effect of substrate concentration on glucose oxidase. 5 μg. notatin, 0·1 ml. catalase, 0·2 M-phosphate buffer pH 5·6. Temp. = 39°.

The influence of O_2 tension is summarized in Fig. 7 from which it is seen that the activity in pure O_2 is nearly 2½ times higher than in air. In these experiments 10 μg. notatin was used. From Fig. 6 the Q_{O_2} of the enzyme at maximum glucose concentration is found to be 82,000 while from Fig. 7 where less glucose was present the figures are 60,000 and 140,000 in air and O_2 respectively. Coulthard *et al.* estimate the Q_{O_2} of their purest enzyme to be 120,000 at 30° in O_2, a figure which is corroborated by our results. As the purity of our sample of notatin was 80–90% the activity of the completely pure enzyme may be expressed by increasing these Q_{O_2} values by about 10%. In comparing these figures with those of previous workers (e.g. Franke & Deffner (1939) reach $Q_{O_2} = 8000$) it should be remembered that the earlier experiments were carried out in O_2 at 30° in absence of catalase. The absence of catalase will tend to increase the O_2 uptake

(Keilin & Hartree, 1945*b*) while the lower temperature will slightly decrease it (see below) hence the old and new figures can be considered as reasonably comparable. Franke & Deffner's preparations were therefore only 5–10 % pure.

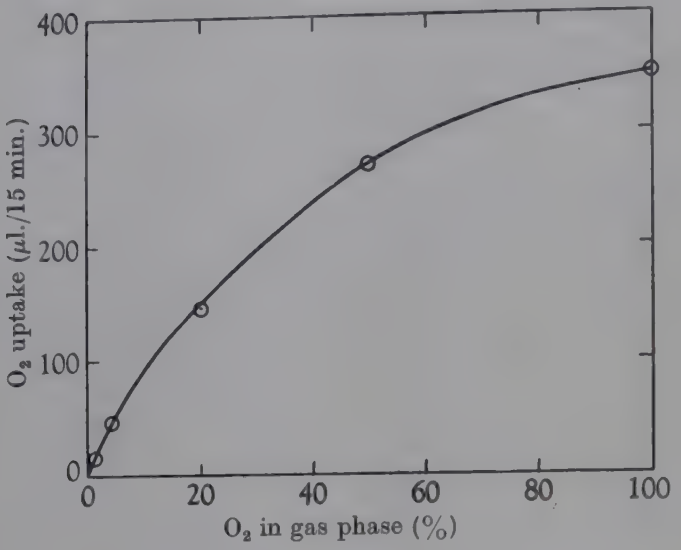

Fig. 7. Effect of O_2 tension on the activity of glucose oxidase. 10 µg. notatin, 1 % glucose, 0·1 ml. catalase, 0·2 M-phosphate buffer pH 5·6. Temp. = 39°.

Effect of temperature. Fig. 8 shows the relationship between enzyme activity and temperature using 12·5 µg. notatin under the standard conditions. The temperature coefficient of the reaction falls off rapidly with rising temperature.

Substrate specificity. In Table 3 are shown the various sugars and sugar derivatives which have been tested with glucose oxidase. The standard conditions for the test were 0·05 M-substrate, 0·1 ml. catalase and 300 µg. notatin. For comparison purposes glucose was tested under the same conditions with 10 µg. notatin from which the O_2 uptake with

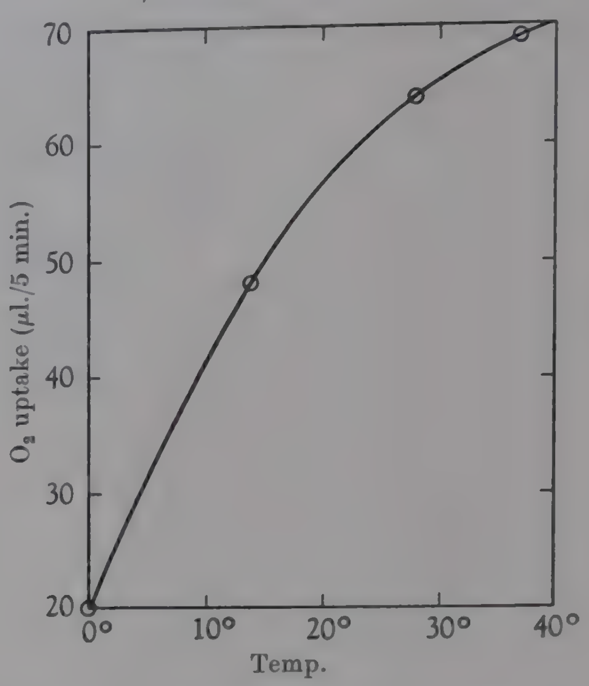

Fig. 8. Effect of temperature on the activity of glucose oxidase. 12·5 µg. notatin, 1 % glucose, 0·1 ml. catalase, 0·2 M-phosphate buffer pH 5·6.

Table 3. *Specificity of glucose oxidase*

Not oxidized

Sugars

Arabinose
Fructose
Fucose
Glucoheptose
Lactose
Melizitose
Raffinose
Rhamnose
Ribose
Sorbose
Sucrose
Tagatose

Methylated sugars

2-Methylglucose
3-Methylglucose
2:3-Dimethylglucose
2:3:6-Trimethylglucose
2:4:6-Trimethylglucose
2:3:4:6-Tetramethylglucose

2:4-Dimethyl-α-galactose
4:6-Dimethyl-α-galactose
2:4:6-Trimethyl-α-galactose

2-Methyl-β-galactose
2:6-Dimethyl-β-galactose
3:4-Dimethyl-β-galactose

3:4:6-Trimethylfructofuranose

Methyl glucosides

α-Methylglucoside
2-Methyl-α-methylglucoside
2:3-Dimethyl-α-methylglucoside
2-Methyl-β-methylglucoside
2:3:6-Trimethyl-β-methylglucoside

Acetyl glucoses

2:3:4:6-Tetraacetylglucose
Pentaacetylglucose
3:4:6-Triacetyl-2-deoxyglucose

Phosphorylated sugars

Glucose-1-phosphate
Hexose-6-phosphate
Fructosediphosphate

Oxidized (glucose = 100)

6-Methylglucose	1·85	Melibiose	0·11
4:6-Dimethylglucose	1·22	Cellobiose	0·09
Mannose	0·98	Glucuronic acid	<0·05
Xylose	0·98	Galacturonic acid	
Trehalose	0·28	2-Aminoglucose	
Maltose	0·19	2-Aminogalactose	
Galactose	0·14		

300 μg. could be calculated. Although the rate of O_2 uptake is not strictly proportional to the quantity of enzyme taken, the figures obtained for the various sugars are nevertheless comparable among themselves. Franke & Deffner (1939), using an enzyme preparation of $Q_{O_2} = 2000$, give the ratio of O_2 uptakes as follows: glucose : mannose : galactose = 1 : 0·07 : 0·14. Similar ratios are recorded by Müller (1941). From Table 3 it is seen that of the commoner sugars, apart from glucose, only mannose and xylose are oxidized though at a rate $\ngtr 1$ % that of glucose. Galactose is much less readily attacked and the previously recorded higher activities are undoubtedly due to the glucose which is invariably present in samples of galactose prepared in the normal way by hydrolysis of lactose. This point is illustrated in Fig. 9, which shows the O_2 uptake under the standard conditions with 300 μg. notatin with a commercial sample of 'pure' galactose, and a specially purified sample kindly provided by Dr D. J. Bell. With all commercial galactoses there is an initial rapid O_2 uptake followed by a slow but steady oxidation of the galactose. By extrapolation to zero time the glucose content may be estimated. Thus in the upper curve of Fig. 9 the glucose content ≡ 80 μl. O_2, i.e. 1·29 mg. or 4·0 %. It is impossible to be certain that all phosphorylated sugars are unattacked as the very slight O_2 uptakes frequently observed may be due to a slow hydrolysis. The slow oxidations of 6-methyl- and 4:6-dimethylglucose, however, appear to be genuine since the rates of oxidation remain constant over long periods.

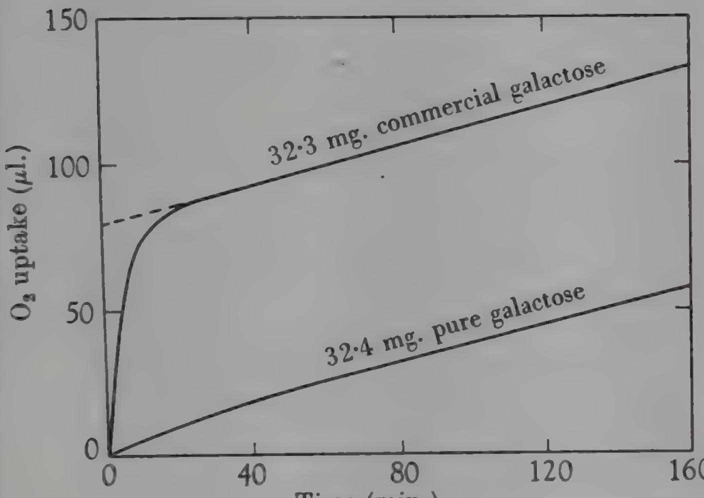

Fig. 9. Oxidation of pure galactose, and commercial galactose containing glucose, by glucose oxidase. 300 μg. oxidase, 0·1 ml. catalase. pH = 5·6, Temp. = 39°.

Inhibitors of glucose oxidase. Working with enzyme preparations of Q_{O_2} 20–30 Franke & Lorenz (1937) found that narcotics (alcohols, urethanes) had a marked inhibitory effect comparable in magnitude to that observed with, for example, succinic dehydrogenase. However, we have found the pure oxidase not to be affected by this class of substances. The respiratory inhibitors HCN, H_2S, HN_3 do not inhibit glucose oxidase, a fact which follows from the flavine nature of the enzyme, but may even give rise to an increased O_2 uptake. This increase is due to the poisoning of the catalase present in all but the purest samples of notatin and the consequent accumulation of H_2O_2. In his earliest experiments Müller (1928) records a slight inhibition of his enzyme by cyanide although as high a concentration as 0·5 M was required in order to obtain 50 % inhibition.

In attempts to find a powerful, specific and reversible inhibitor of glucose oxidase we have tested a large range of substances but without positive results. In a few cases a very slight inhibition was observed. Thus under the standard conditions 0·01 M concentrations of 8-hydroxyquinoline, sodium nitrate and semicarbazide gave rise to 11, 13 and 20 % inhibition respectively.

Oxidation of glucose by Penicillium notatum. The mould (strain 4222 of the National Collection of Type Cultures) was grown in plugged Barcroft manometer flasks on 5 ml. Czapek-Dox medium (Coulthard *et al.* 1945). After 3–10 days growth at 24° the medium was sucked off and replaced with successive lots of 0·2 M-phosphate buffer pH 5·6. In this way all traces of the medium were removed. The respiration

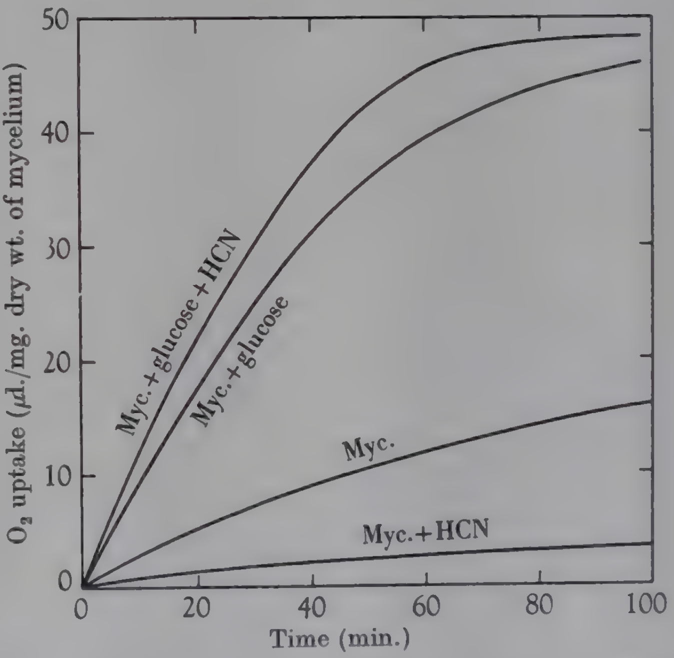

Fig. 10. Oxidation of glucose by washed 10-day mycelia (Myc.) of *Penicillium notatum*. Effect of cyanide. 2 % glucose, 0·004 M-HCN, 0·2 M-phosphate buffer pH 5·6. Temp. = 39°. The dry weights of mycelia, grown in manometer flasks, were 21–25 mg.

of the mycelia was tested at pH 5·6 in presence of 2 % glucose and/or 0·002 M-HCN. The centre wells of the manometers contained a roll of filter paper soaked in 10 % NaOH or, when cyanide was present, with the appropriate NaOH-KCN mixture (Umbreit Burris & Stauffer, 1945). The results of such experiments with a 10-day culture are shown in Fig. 10. The O_2 uptakes are given for mg. dry weight of mycelium, the dry weight of the latter (21–25 mg.) being determined at the end of the experiment. Experiments with a 3-day culture, showing a much higher Q_{O_2}, gave essentially the same results. Although the endogenous respiration is cyanide-sensitive, the oxidation of glucose is not. Hence it must be accepted that the intracellular oxidation of glucose to gluconic acid, like the extracellular reaction, does not involve the cytochrome system.

Cultivation of Penicillium notatum *on a fructose medium.* An attempt to affect the specificity of glucose oxidase was made by repeated subcultivation on a Czapek-Dox medium in which glucose had been replaced by fructose. Small plugged test tubes were used, each containing 1 ml. medium. The organism grew well at 24°, and was subcultivated every 4 days within which time a thick crop of spores was produced under these conditions. After 20 passages the culture was inoculated into two culture dishes, 15 cm. diameter, each containing 200 ml. of the fructose medium. Ten days later the yellow culture medium was removed and tested in the usual way with glucose and fructose. It was found to be still completely specific for glucose.

SUMMARY

1. Mould glucose oxidase of Müller obtained in the highly purified state (notatin) by Coulthard *et al.* from *Penicillium notatum* was investigated in respect to its structure, physical properties and enzyme kinetics.

2. The enzyme, as was previously shown, catalyzes the oxidation of glucose to gluconic acid by molecular O_2, which is reduced to H_2O_2

$$(C_6H_{12}O_6 + H_2O + O_2 = C_6H_{12}O_7 + H_2O_2).$$

3. The enzyme, which is a flavoprotein, shows in the oxidized state the characteristic absorption spectrum with bands at 377 and 455 mμ. On addition of glucose the enzyme is almost completely decolorized and the bands become hardly perceptible. They disappear completely in presence of $Na_2S_2O_4$. That the prosthetic group is the alloxazine-adenine dinucleotide was demonstrated by removing the prosthetic group from D-amino-acid oxidase, and regenerating the activity of the latter by the addition of boiled notatin.

4. From the absorption constant at 455 mμ. the minimum molecular weight is estimated to be about 75,000. The molecular weight calculated from the sedimentation velocity and diffusion constant is 152,000 (see addendum), which shows that the molecule of enzyme contains two prosthetic nuclei.

5. From the behaviour of the most active enzyme preparation in the ultracentrifuge and in the Tiselius electrophoresis apparatus, it would appear to be between 80 and 90 % pure.

6. The enzyme does not fluoresce in ultraviolet light within the pH limits of its catalytic activity (pH 2–8). Outside this range it loses its activity and becomes fluorescent.

7. The enzyme shows a pronounced specificity for glucose. Of about 50 sugars and their derivatives tested, four are oxidized at about 1 % of the rate of glucose oxidation (mannose, xylose, 6-methylglucose and 4:6-dimethylglucose), whereas the remainder are virtually or completely unattacked. The previously recorded oxidations of galactose are due to glucose impurities.

8. In experiments with mycelia of *P. notatum* grown in manometer flasks it was found that the endogenous respiration of the mycelium was cyanide-sensitive, but that the oxidation of glucose by the mycelia was not inhibited by cyanide. Thus the cytochrome system is not involved in the intracellular oxidation of glucose to gluconic acid.

9. Attempts to modify the specificity of glucose oxidase by repeated subcultivation on a fructose medium were unsuccessful.

The authors wish to thank Prof. H. Raistrick, Dr J. H. Birkinshaw and Dr W. F. Short for generous gifts of highly purified notatin, Dr D. J. Bell and Prof. M. Stacey for numerous samples of sugars, and Dr M. Dixon for facilities in the use of the Beckman photoelectric quartz spectrophotometer and the Tiselius electrophoresis apparatus.

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Addendum. Sedimentation and Diffusion of Glucose Oxidase (Notatin)

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A sample of glucose oxidase (Coulthard *et al.* 1945) was examined with the Svedberg oil-turbine ultracentrifuge by the method of Philpot (1938), and its diffusion constant was measured by a new method (Coulson, Cox, Ogston & Philpot, 1948). There was a main sedimenting component (Fig. 1 (*a*)) which accounted for at least 80% of the total refracting material and which appeared to be homogeneous: in addition, there were small quantities of a faster and a slower component (Fig. 1 (*b*) and (*c*)). The whole of the absorption at 495–515 mμ. was associated with and sedimented at the same speed as the main component. The buffer was NaCl 0·2 M; KH_2PO_4 0·057 M; NaH_2PO_4 0·0095 M: the speed of rotation was 1015 rev./sec. The results are given in the table.

The values of M and of f/f_0 are obtained assuming a value of 0·75 for the partial specific volume of the protein. The value of D_{20} is an average for all the protein present; since this is not strictly monodisperse, the value may not be accurate for the main component. However, the value of M is in reasonable agreement with that of the equivalent weight as estimated in the preceding paper.

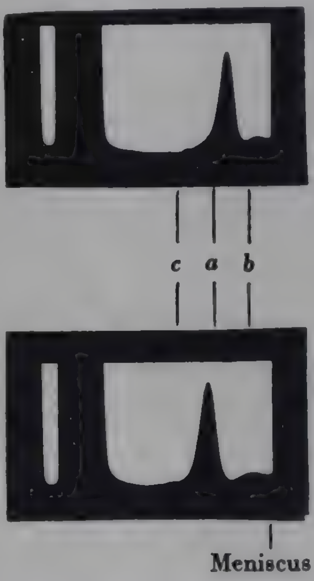

Fig. 1. Diagrams of glucose oxidase boundaries 15 and 25 min. after reaching 1015 rev./sec.

Refractive increment ×10⁴	Concentration (g./100 ml.)	S_{20} $\times 10^{13}$	S_{20} (corr.) $\times 10^{13}$	D_{20} $\times 10^{7}$	M	f/f_0
13·5	0·75	7·25	7·86	—	—	—
6·4	0·36	7·62	8·27	5·13	152,000	1·16

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The Use of Glucose Oxidase (Notatin) for the Determination of Glucose in Biological Material and for the Study of Glucose-producing Systems by Manometric Methods

By D. KEILIN and E. F. HARTREE, *Molteno Institute, University of Cambridge*

(*Received* 7 *July* 1947)

Glucose oxidase or notatin, obtained in a highly purified state from culture media of *Penicillium notatum* (Coulthard, Michaelis, Short, Sykes, Skrimshire, Standfast, Birkinshaw & Raistrick, 1945), catalyzes the aerobic oxidation of glucose to gluconic acid. The fact that this enzyme shows both a high affinity and a marked specificity for glucose (see preceding paper) suggested the possibility of its utilization for the estimation of glucose in presence of other sugars or of proteins, and for the study of the kinetics of different enzyme reactions which involve the liberation of glucose from its derivatives such as di- and polysaccharides, phosphorylated sugars and glucosides. Manometric methods, owing to their rapidity and simplicity, have great advantages over other methods of analysis on a micro scale and have been applied, for example, to the estimation of urea by urease, glutathione by glyoxalase, succinic acid by muscle preparations and amino-acids by specific bacterial decarboxylases. An outstanding merit of such methods is that they can be made far more specific than any purely chemical methods. The use of notatin extends the application of manometric methods to all reactions which involve the liberation of glucose.

In this paper we shall describe various examples of the use of notatin for the manometric estimation of glucose, and for manometric studies of the kinetics of reactions catalyzed by different enzymes.

EXPERIMENTAL

If the amount of glucose present in a solution is being determined, the notatin must be added from dangling tubes when the manometers have been equilibrated in the thermostat. On the other hand, in studying the rate of production of glucose by a hydrolytic enzyme either this enzyme or its substrate (e.g. a glucoside) should be added from the dangling tube in order that glucose production may be followed in terms of O_2 uptake from the beginning of the reaction. It is important in all experiments of the latter type that the notatin should be present in excess, and that the rate of production of glucose should be the limiting factor in the overall reaction. Under such conditions there will be no accumulation of glucose in the reaction mixture and the rate of O_2 uptake catalyzed by notatin will be a genuine measure of glucose production, i.e. of the activity of the hydrolytic enzyme.

The oxidation of glucose by the highly purified glucose oxidase proceeds quantitatively and the O_2 uptake under specified conditions can be taken as a measure of the glucose present (Keilin & Hartree, 1945*a*). The primary oxidation reaction is the formation of gluconic acid and H_2O_2:

$$C_6H_{12}O_6 + O_2 + H_2O = C_6H_{12}O_7 + H_2O_2. \qquad (1)$$

When using very pure glucose oxidase (notatin) in slightly acid solution (the optimum pH is 5·6) the H_2O_2 is not decomposed at an appreciable rate, and the final value of the O_2 uptake will be 1 mol. O_2/mol. glucose (124·4 μl. O_2/mg. glucose). As a general rule part of the H_2O_2 decomposes, and in order to estimate glucose by a manometric technique it is essential to add catalase which will decompose the H_2O_2 as it is formed. The nett reaction is then

$$C_6H_{12}O_6 + \tfrac{1}{2}O_2 = C_6H_{12}O_7, \qquad (2)$$

and the O_2 uptake will be 62·2 μl./mg. glucose. We have shown previously that the addition of certain alcohols to an enzyme system producing H_2O_2 will, in presence of catalase, lead to an oxidation of the alcohol to the aldehyde (Keilin & Hartree, 1936*a*, 1945*a*). This coupled oxidation of the alcohol is catalyzed by catalase. The overall reaction is now

$$C_6H_{12}O_6 + O_2 + R.CH_2OH = C_6H_{12}O_7 + H_2O + R.CHO, \qquad (3)$$

and, if the alcohol is present in excess, the O_2 uptake again reaches the value of 124·4 μl./mg. glucose.

It is important in all quantitative estimations of O_2 uptake to ascertain that under the experimental conditions the H_2O_2 formed by reduction of O_2 is either quantitatively decomposed into water and O_2 or completely utilized in a coupled oxidation. Under either of these conditions there is a stoicheiometric relationship between the glucose oxidized and the O_2 uptake of the system. In absence of any substances which can undergo coupled oxidation in presence of H_2O_2 and catalase the former will be quantitatively decomposed into water and O_2, but in practice part of the H_2O_2 will as a rule be utilized in coupled oxidations. It is therefore advisable in all cases to investigate the effect on the system of ethanol, the presence of which will ensure that any H_2O_2 not utilized for other coupled oxidations will be utilized for oxidation of ethanol to aldehyde.

Whereas in our experiments we have made use of pure glucose oxidase, satisfactory results can be obtained with crude material and even with culture media of *P. notatum* obtained as described by Coulthard *et al.* (1945). Such enzyme preparations will naturally be more liable to exhibit coupled oxidations than the pure material.

Detergents and proteolytic enzymes are sometimes used for the preparation of tissue extracts suitable for analysis, and experiments were carried out in order to determine their effect upon notatin. We have found that after 1 hr. incubation at 39° and pH 5·6 with 0·2% sodium deoxycholate, 0·2% sodium dodecylsulphate, 1% pepsin or 1% papain (in presence of cysteine) the catalytic activity of notatin was unimpaired.

Oxygen uptakes were measured in Barcroft differential manometers, usually at 39°. The flasks contained a total volume of 3·3 ml., one of the components of the system being added from a dangling tube so that the complete O_2 uptake from zero time could be recorded. Whenever possible the pH of the reaction mixture was at or near 5·6, although any pH between 3·5 and 7 is permissible. The highly purified sample of notatin (see preceding paper) was made up as a 0·1% aqueous solution which was stable for 1 week at 5°. Catalase was obtained from horse liver by the method previously described (Keilin & Hartree, 1945*b*).

Estimation of glucose in blood plasma

Samples of oxalated blood of a rat (A) or of cats (B, C, D) were centrifuged immediately after removal from the animal and each sample was tested in two manometers. The right-hand flask of one manometer received 2 ml. plasma, 0·1 ml. catalase and buffer (0·25M-phosphate, pH 5·6) to 3·0 ml. while the second manometer received the same ingredients plus 0·1 ml. 20% (v/v) ethanol. In each flask was placed a dangling tube containing 0·3 ml. notatin solution (300 µg.). Both left-hand flasks received 3·3 ml. buffer. After equilibration in a thermostat at 39° the dangling tubes were dislodged and the O_2 uptakes followed manometrically. In presence of ethanol the H_2O_2 formed during the oxidation of glucose should be quantitatively utilized for oxidation of ethanol according to equation (3). In absence of ethanol the reaction should follow equation (2) unless there are in plasma oxidizable substances which can take the place of ethanol in coupled oxidation.

Fig. 1 shows the results obtained with plasma A. In absence of ethanol, 1 mg. glucose ≡ 62·2 µl. O_2 and the final O_2 uptake corresponds to 3·63 mg. glucose. In presence of ethanol 1 mg. glucose ≡ 124·4 µl. O_2 and the O_2 uptake corresponds to 3·37 mg. glucose. In all cases the experiments without ethanol gave O_2 uptakes slightly more than half that observed in presence of ethanol. In Table 1 are summarized the results obtained with the 4 samples of plasma together with figures for reducing sugar estimated by the Hagedorn & Jensen method (1923). The latter method gives results which are

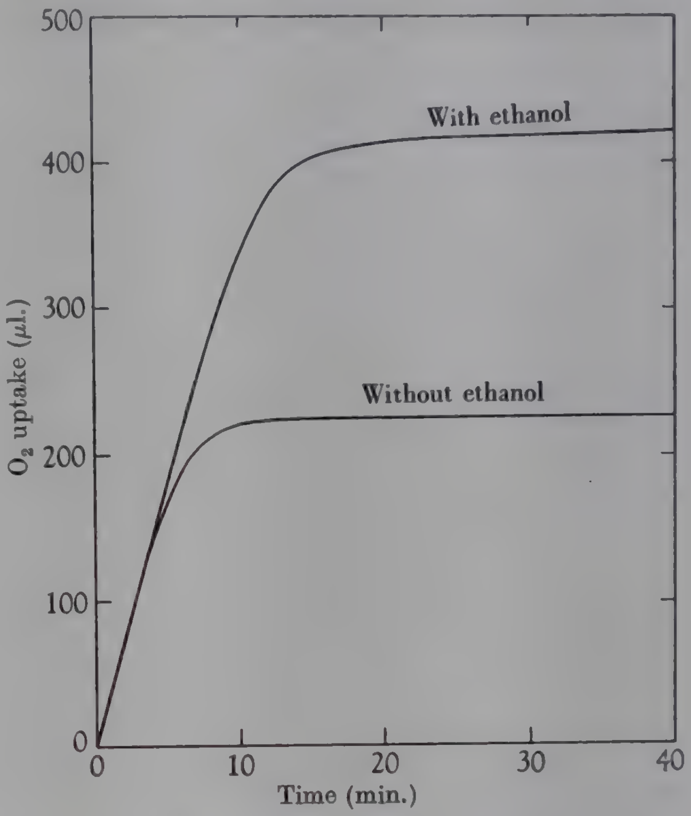

Fig. 1. Oxidation by notatin of the glucose in 2 ml. rat plasma in presence and in absence of 20 mg. ethanol. Each right-hand flask contains 300 µg. notatin, 0·1 ml. catalase, 2 ml. plasma and 0·1M-phosphate buffer pH 5·6. Temp. = 39°.

Table 1. *Estimation of glucose in blood plasma (a) manometrically with notatin + catalase ± ethanol (see Fig. 1 for details) and (b) by the Hagedorn & Jensen method*

	Glucose found by manometric methods (mg.)		Hagedorn & Jensen estimation of glucose (mg.)		
	− Ethanol	+ Ethanol	Total reducing substances as glucose	Non-fermentable as glucose	Glucose (corrected)
2 ml. plasma A (rat)	3·63	3·37	4·05	—	—
2 ml. plasma B (cat)	4·40	4·06	4·73	—	—
2 ml. plasma C (cat)	3·20	2·72	3·45	0·63	2·82
2 ml. plasma D (cat)	3·53	2·99	3·67	0·66	3·01

higher than those obtained by either manometric method. However, in two cases a correction was made for the non-fermentable reducing substances in the plasma by estimating the apparent glucose content before and after fermenting the glucose by means of yeast. The procedure described by Mann (1946) was used for this purpose. When this correction was applied the agreement between the Hagedorn & Jensen method and the manometric method in presence of ethanol was very satisfactory.

Estimation of glucose in other biological media

The method just described for blood plasma can be applied generally to plant and animal fluids or tissue extracts. In the case of some plant extracts (e.g. potato) it is necessary to destroy the phenol-oxidases which, in presence of the natural substrates in the extracts, give rise to a rapid O_2 uptake with consequent formation of highly coloured oxidation products. The enzyme is rapidly destroyed by heating to 80° for 10–15 min., and after such treatment the O_2 uptake of the extract alone will be very small and can be applied as a correction to the uptake in presence of notatin. Tests have been carried out on cabbage sap and on press juices from potato, beetroot and mangold, and in each case the extract was tested (*a*) with notatin and catalase and (*b*) with notatin, catalase and ethanol, at 39° under the above conditions. The results are summarized in Table 2. As in the case of plasma it is seen that higher values are obtained in absence of ethanol owing to a partial utilization of H_2O_2 for coupled oxidation. The results in presence of ethanol are therefore the more reliable. By carrying out an estimation in presence of invertase, both glucose and sucrose may be determined, the difference in O_2 uptakes in presence and in absence of invertase, being a measure of the glucose equivalent of the sucrose. In such experiments the O_2 uptake reaches a steady value in 20–30 min.

Table 2. *Glucose estimation in various plant extracts using notatin*

(300 μg. notatin, 0·1 ml. catalase, 0·1 M-phosphate buffer pH 5·6. Temp. = 39°. O_2 uptakes shown are the final steady values.)

	Plant juice alone	Plant extract + notatin + catalase*		Plant extract + notatin + catalase + ethanol†		Plant extract + notatin + catalase + ethanol + invertase†	
System in manometer	O_2 uptake (μl.)	O_2 uptake (μl.)	Glucose (mg.)	O_2 uptake (μl.)	Glucose (mg.)	O_2 uptake (μl.)	Sucrose (mg.)
2 ml. cabbage sap	0	286	4·60	495	3·97	—	—
1 ml. potato press juice (heated)	7	173	2·64	308	2·42	—	—
0·5 ml. beetroot press juice	3	179	2·83	323	2·57	538	3·28
0·5 ml. mangold press juice	0	245	3·94	391	3·14	1048	10·05

* 1 mg. glucose ≡ 62·2 μl. O_2 (see p. 230). † 1 mg. glucose ≡ 124·4 μl. O_2 (see p. 230).

By the manometric method as little as 0·1–0·2 mg. glucose may be accurately estimated. The main advantages of the method are its simplicity, the fact that it is not necessary to remove proteins and, above all, the high specificity of notatin for glucose. On the other hand the method requires about 10 times the quantity of material necessary for the Hagedorn & Jensen method. Furthermore, the manometric method cannot be used for analysis of whole blood as part of the oxyhaemoglobin will become oxidized to methaemoglobin with consequent liberation of the bound O_2. When the quantity of glucose is too small for manometric estimation notatin may be utilized in connexion with the Hagedorn & Jensen method. Thus by estimation of reducing substances by the latter method, before and after incubation with notatin, the glucose may be accurately estimated. This was done by Mann (1946) who has shown that the reducing values of seminal plasma before and after treatment with glucose oxidase were approximately the same. In this way he demonstrated the virtual absence of glucose in the seminal plasma, and confirmed his discovery that the main reducing carbohydrate of seminal plasma is D-fructose.

Studies on invertase

(*a*) *Activity of invertase* in vitro. The standard methods for the study of invertase involve the use of the polarimeter or the periodic acid estimation of reducing sugar. The former gives a continuous record, but cannot be applied to solutions which are cloudy or which contain large quantities of other optically active substances. The latter method only gives the quantity of sucrose inverted at one instant during the reaction, and becomes laborious when continuous records of a number of experiments are required. Neither of these difficulties arise when the reaction is carried out in presence of notatin and the glucose production followed by the O_2 uptake in a manometer. As a source of enzyme British Drug Houses 'invertase concentrate' was used at a dilution of 1 in 250 and the manometric method was checked by estimation of reducing sugar at the end of the experiment.

Two Barcroft manometers were filled as follows: The right-hand flask of each manometer received 0·3 ml. diluted invertase, 0·6 ml. 0·1% notatin, 0·1 ml. catalase, 2·0 ml. 0·25 M-phosphate pH 5·6 and 0·3 ml. 10% sucrose in a dangling tube. The left-hand flasks received 3·3 ml. buffer. The experiments were carried out at 20°, and the sucrose was added after equilibration and closing the manometer taps. One manometer was shaken for 28 min. from the addition of sucrose when the O_2 uptake was 312 μl. At 29 min. 1 ml. liquid from the right-hand flask was added to a hot mixture of 25 ml. 0·45% $ZnSO_4.7H_2O$ and 5 ml. 0·1 N-NaOH, heated at 100° for 3 min. and filtered. The reducing sugar was estimated in the filtrate by the Hagedorn & Jensen method. The second manometer showed an O_2 uptake of 470 μl. at 42 min. At 43 min. the reducing sugar in the left-hand flask was similarly estimated. The O_2 uptakes for 29 and 43 min. were calculated from the observed values at 28 and 42 min. respectively, and the results obtained by the two methods are shown in Table 3 where the glucose found manometrically is seen to be very close to the residual reducing sugar (fructose).

Table 3. *Invertase activity measured (a) manometrically with notatin, and (b) by estimation of reducing sugar*

(0·3 ml. invertase (B.D.H. concentrate 1 : 250), 600 μg. notatin, 0·1 ml. catalase, 30 mg. sucrose. 0·15 M-phosphate pH 5·6. Temp. = 20°.)

	In 29 min.	In 43 min.
O_2 uptake (μl.)	312	470
Glucose equivalent (mg.)	5·01	7·57
Residual reducing sugar = fructose (mg.) by Hagedorn & Jensen method	5·13	7·40

In Fig. 2 is shown the effect of varying concentrations of sucrose on the activity of an invertase solution at 17° and pH 5·6. Nelson & Sottery (1924) have shown that low concentrations of glucose activate, and higher concentrations inhibit the initial velocity of hydrolysis of sucrose by honey-bee salivary invertase. The rate of inversion was, on the other hand, only slightly affected by fructose. The effect of high concentrations of fructose or invertase could not be readily determined by the usual methods, but by means of notatin it could be shown manometrically that 0·167 M-fructose inhibits the rate of hydrolysis of 0·007 M-sucrose in presence of invertase by only 18%. The study of the kinetics of the invertase reaction by means of notatin is simplified owing to the complete elimination of glucose which may either accelerate or retard the reaction.

(*b*) *Activity of invertase* in vivo. Yeasts contain an exceptionally powerful invertase which, from our preliminary experiments, appeared to be far more active than would be necessary to maintain a sufficient supply of monosaccharide for normal metabolic requirements. It therefore seemed probable that in presence of sucrose, respiring yeast cells would discharge large quantities of invert sugar into the surrounding medium. The yeast selected for this work was *Torulopsis utilis* (National Collection of Type Cultures no. 2576). This yeast does not ferment sugars under aerobic conditions, and it therefore becomes a simple matter to compare its metabolic requirements with the activity of the intracellular invertase by a simple manometric technique using notatin.

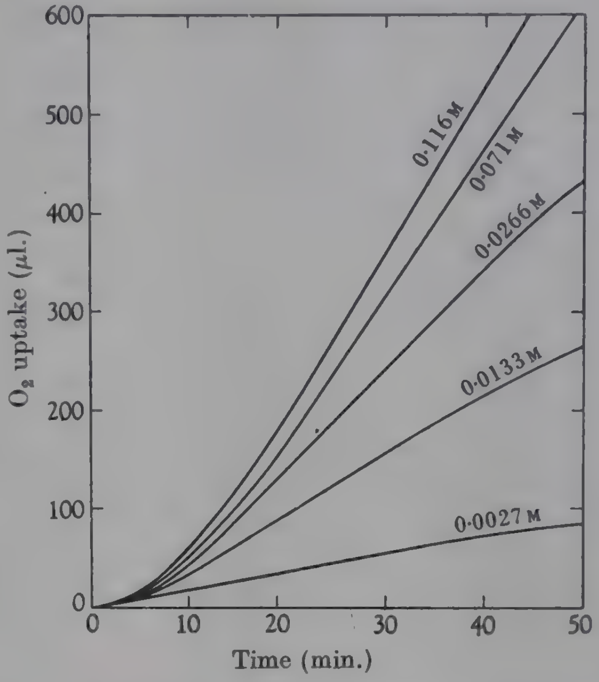

Fig. 2. Inversion of varying concentrations of sucrose by invertase followed manometrically by oxidation of the liberated glucose by notatin. Each right-hand flask contains 0·2 ml. invertase concentrate (1 : 250), 500 μg. notatin, 0·1 ml. catalase, 0·2 M-phosphate buffer pH 5·6. Figures express concentration of sucrose. Temp. = 17°.

The organism was grown in Roux flasks on beer wort containing 3% agar and adjusted to pH 6·5. The flasks containing the medium were sterilized for 20 min. at 100° on three successive days. After inoculation the flasks were incubated at 30° for 2 days and then at room temperature for 3 days. The cells were collected in distilled water and washed repeatedly with distilled water in centrifuge tubes. A suspension in water was prepared containing 0·2 mg. dry wt./ml. Table 4 shows the ingredients of the right-hand flasks of the four manometers used for the test. The left-hand flasks received 3·0 ml. buffer and a roll of filter paper soaked in 10% NaOH.

In view of the very high invertase activity as little as 0·1 mg. dry weight of the yeast is sufficient. The Q_{O_2} was estimated separately on a larger quantity of yeast (Fig. 3). After closing the taps the manometers were shaken for 15 min. prior to addition of sucrose from the dangling tubes: during this period the O_2 uptake was negligible. At

15 min. the sucrose was added and the O_2 uptake followed for the next 55 min. (Fig. 3). At this point the reaction was stopped by addition to each right-hand flask of a hot mixture of 1 ml. 4·5% $ZnSO_4.7H_2O$, 0·2 ml. N-NaOH and 6 ml. H_2O. The quantities of reducing sugars, estimated as already described, in the protein-free filtrates, are given in Table 4 together with the corresponding O_2 uptakes. The residual reducing sugar in manometer 1 = invert sugar formed minus sugar oxidized by yeast, and this value must be corrected by the figure from manometer 4 which represents the reducing value of the yeast. Similarly the reducing sugar formed in manometer 2 represents sugar inverted minus sugar oxidized by yeast and notatin, and this figure must be similarly corrected from manometer 3.

Table 4. *Invertase activity of* Torulopsis utilis *determined (a) manometrically with notatin, and (b) by estimation of reducing sugars in presence and in absence of notatin*

(The volumes in the right-hand flasks of the manometers were made up to 3 ml. with phosphate buffer (0·25 M, pH 5·6) and the central tube received 0·3 ml. 10% NaOH and a roll of filter paper. The left-hand flasks received 3 ml. buffer and a roll of filter paper soaked in 10% NaOH.)

Components of system (right-hand flask)	Manometer no. 1	 2	 3	 4
0·5 ml. *T. utilis* (0·1 mg.)	+	+	+	+
300 μg. notatin + 0·1 ml. catalase	−	+	+	−
0·3 ml. 10% sucrose in dangling tube	+	+	−	−
O_2 uptake in 55 min. (μl.)	9	250	0	0
Total reducing sugar at 55 min. (mg.)	8·63	4·83	0·02	0·02

Hence glucose oxidized = (1 − 4) − (2 − 3) = 3·80 mg. From the O_2 uptake in manometer 2 the glucose content is 250/62·2 = 4·01 mg. A correction must be applied for the slow oxidation of inverted sucrose (9 μl. O_2) detected in manometer 1. The complete oxidation of 1 mol. invert sugar by yeast requires 12 atoms oxygen instead of the 1 atom utilized by notatin. The correction to be applied to the O_2 uptake in manometer 2 is therefore $9/x$ where x may be as high as 12. The correction is within experimental error, and the glucose oxidized according to the manometric method may be taken as 4·00 mg. This value may be too high for two reasons: (*a*) The sucrose used in these experiments showed a slight reducing action in the Hagedorn-Jensen estimation (= 0·06 mg./manometer) and part at least of this impurity may be glucose, (*b*) a slight coupled oxidation of substances in the yeast or the sucrose may take place (as in the case of the estimation of glucose in plasma (Table 1)). The agreement between the two methods is therefore satisfactory.

From the above results it is seen that 0·1 mg. *T. utilis* produces 2 × 3·8 mg. invert sugar in 55 min., i.e. 1 mg. produces 83 mg./hr. The complete oxidation of this quantity of sugar by the organism would require 83 × 62·2 × 12 = 62,000 μl. O_2. In other words if the yeast could oxidize the monosaccharides to CO_2 and H_2O as rapidly as they were produced its Q_{O_2} would be 62,000 instead of 112, the figure obtained experimentally (Fig. 3). There is thus a considerable 'overflow' of invert sugar.

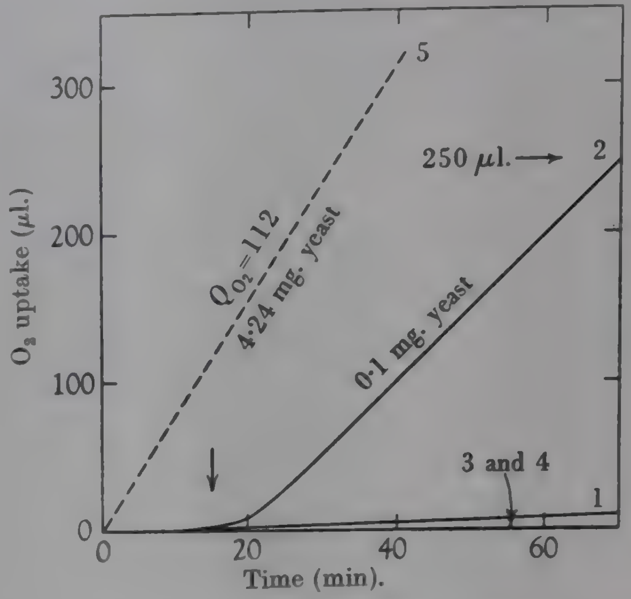

Fig. 3. Inversion of sucrose by yeast (*Torulopsis utilis*) followed manometrically in terms of oxidation of the liberated glucose by notatin. Experiment carried out at 39° in four manometers (1–4, see Table 4). Each manometer receives 0·1 mg. (dry wt.) *T. utilis* in 0·1 M-phosphate buffer pH 5·6; manometers 2 and 3 also contain 300 μg. notatin and 0·1 ml. catalase. 0·3 ml. 10% sucrose was added after 15 min. to manometers 1 and 2. Manometer 5: O_2 uptake by 4·24 mg. yeast in presence of 0·3 ml. 10% sucrose. The central tubes of all flasks receive 0·3 ml. 10% NaOH and a roll of filter paper. The numbering of the curves corresponds to that of the manometers.

Experiments similar to those just described have been carried out on baker's yeast, brewer's yeast, *B. subtilis* and mycelia of *P. notatum* (see preceding paper). The first 3 organisms showed a similar large 'overflow' of invert sugar, but *P. notatum* was unable to invert sucrose.

Maltase

The study of maltase activity by standard methods is straightforward since only 2 sugars are present in the reaction mixture, viz. maltose and glucose. Nevertheless the activity of the enzyme is most conveniently studied with the help of notatin. As a source of maltase we have used a Lebedew juice prepared by dispersing 50 g. air-dried brewer's yeast in 150 ml. water, incubating 3 hr. at 39° and filtering. In presence of catalase and 300 μg. notatin, 0·5 ml. Lebedew juice showed, under the experimental conditions already described for invertase, a very slow O_2 uptake (40 μl./hr.). On addition of maltose O_2 uptakes up to 1500 μl./hr. were observed. It was of interest to find that the maltase activity of living brewer's yeast was insufficient to provide any 'overflow' of glucose which could be detected by means of notatin when the yeast was shaken in a manometer with maltose. The invertase activity of the brewer's yeast, however, was such as to provide a very large 'overflow' (see above).

Amylases

These hydrolyzing enzymes may be divided into two classes: the α-amylases present in pancreas, saliva, micro-organisms and malt, which break down starch into dextrins of mol. wt. 1000–3000 and finally into maltose and glucose, and the β-amylases of ungerminated seed grains which yield dextrins and β-maltose but no glucose (Hopkins, 1946). The formation of glucose and maltose from starch by α-amylases has been studied by Somogyi (1938, 1940) and by Myrbäck (1941, 1942). While the latter has found evidence that malt amylase produces the two sugars simultaneously, the former considers that maltose precedes glucose in the reaction catalyzed by α-amylases from animal sources, and that the final quantities of maltose and glucose produced correspond to about 60 and 10% respectively of the total starch. Such investigations are hampered by the complexity of the mixtures of breakdown products and by difficulties in the determination of glucose and maltose in mixtures. The preferential fermentation of glucose by baker's yeast at pH 8·4, though far from satisfactory, has until now been the only method of determining glucose in presence of maltose. The value of notatin in such investigations is obvious and we have been able to demonstrate manometrically the formation of glucose not only from soluble starch, but also from glycogen using both saliva and an α-amylase isolated from saliva by the method of Hanes & Cattle (1938). The manometer flasks contained 300 μg. notatin, 0·1 ml. catalase, 50 mg. carbohydrate and a suitable amount of amylase. The final concentration of phosphate buffer (pH 5·6) was 0·1 M and the experiments were carried out at 39°. The O_2 uptakes are plotted in Fig. 4 where curves 1 and 2 show the formation of glucose in presence of 0·5 ml. saliva from glycogen and soluble starch respectively. Curves 3 and 4 represent corresponding experiments with 6 mg. amylase. In both cases there was no O_2 uptake in presence of maltase after a correction had been applied for the slow rate of oxidation of maltose (see preceding paper). Furthermore the polysaccharides themselves were shown to be free from glucose. These experiments offer proof of the accepted view that glucose arises from the splitting of α-1, 4 linkages in the polysaccharides and not from hydrolysis of maltose. At the same time they demonstrate a method for the study of the kinetics of glucose production by amylases; a problem to which there has hitherto been no satisfactory experimental approach. The similarity in the course of the reactions with the two polysaccharides is very striking.

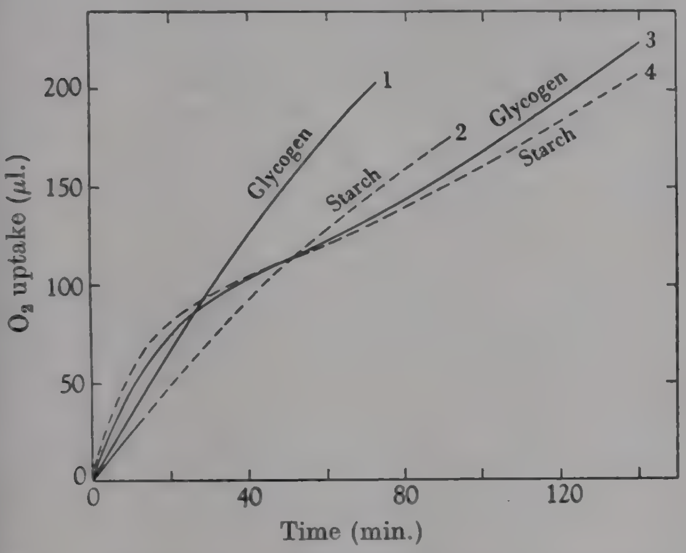

Fig. 4. Oxidation by notatin of the glucose produced from starch and glycogen by saliva and by salivary amylase. Temp. = 39°. All manometers contain 300 μg. notatin, 0·1 ml. catalase and 50 mg. polysaccharide in 0·1 M-phosphate pH 5·6. Manometers 1 and 2 contain 0·5 ml. saliva; manometers 3 and 4 contain 6 mg. amylase. The numbering of the curves corresponds to that of the manometers.

Hydrolysis of glucose phosphates

The phosphatases which hydrolyze glucose-1-phosphate (Cori ester) and glucose-6-phosphate (Robison ester) are widely distributed in animal tissue. A convenient source of enzyme is the intestinal mucosa and a crude preparation was obtained as follows: the small intestine of a rabbit was well irrigated with tap water, the mucosa scraped off and ground in a mortar with an equal volume of acetate buffer (0·1 M, pH 5·6) and sand. The suspension was decanted from the sand after standing 5 min. The standard method of studying the enzyme hydrolysis of these esters is to determine at intervals the increase in inorganic phosphorus. On addition of notatin to the system, however, a continuous manometric record of glucose production can readily be obtained.

For the study of the Robison ester three manometers were filled as shown in Table 5, the notatin being dissolved in 0·3 ml. water and placed in a dangling tube. After equilibration and closing the taps, the notatin was added. The course of the reaction is shown in Fig. 5 (full lines). At 30 min. the manometer readings were taken, giving the O_2 uptakes shown in Table 5, and the reactions stopped by the addition of 3 ml. 20% trichloroacetic acid to each right-hand flask. After standing 1 hr. the mixtures were filtered and the filtrate and washings made up to 25 ml. Inorganic P was estimated by the Fiske-Subbarow method (Rona, 1929). The increase in inorganic P due to phosphatase action is the P content of manometer 1 less that of 2 and 3, i.e. 778 − 32 − 154 = 592 μg. P. The O_2 equivalent of the liberated P is similarly, from Table 5, 221 − 13 − 0 = 208 μl. which corresponds 31 × 208/11·2 = 577 μg. P. The figures obtained by the two methods are in good agreement.

Table 5. *Hydrolysis of Robison ester (glucose-6-phosphate) by a suspension of rabbit intestinal mucosa*

(Volumes made up to 3·3 ml. with 0·2 M-acetate buffer pH 5·6.)

Components of system (right-hand flask)	Manometer no. 1	2	3
1 ml. mucosa	+	−	+
300 μg. notatin + 0·1 ml. catalase	+	+	−
1 ml. Robison ester (0·1 M)	+	+	−
O_2 uptake in 30 min. (μl.)	221	0	13
Inorganic P at 30 min. (μg.)	778	32	154

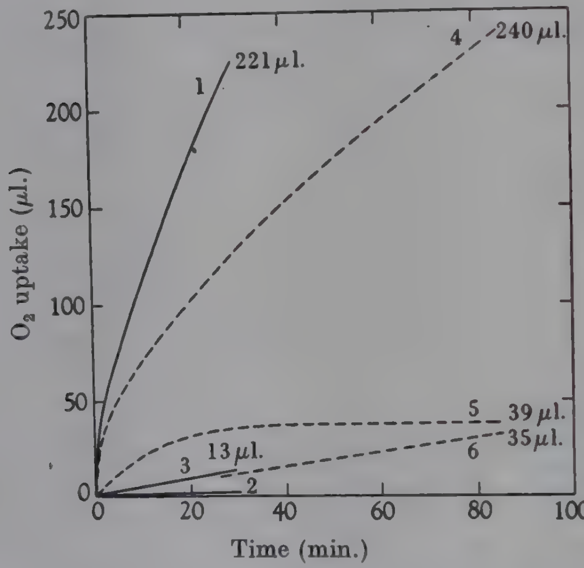

Fig. 5. Rates of hydrolysis at 39° of Robison ester (——) and of Cori ester (- - - -) catalyzed by a phosphatase preparation from intestinal mucosa. Glucose production followed by means of O_2 uptakes in presence of notatin. 0·1 M-acetate buffer pH 5·6. Curves 1 and 4: phosphatase, notatin, catalase and ester. Curves 2 and 5: notatin, catalase and ester. Curves 3 and 6: phosphatase only. For quantities see Tables 5 and 6.

A parallel investigation of the hydrolysis of Cori ester gave the results summarized in Table 6 and Fig. 5 (dashed lines). The hydrolysis of this ester is slower and the sample contained a considerable quantity of inorganic P. The results obtained after allowing the reactions to proceed for 85 min. are as follows: increase in inorganic P = 1298 − 363 − 493 = 442 μg. P. Glucose liberated ≡ 240 − 39 − 35 = 166 μl. O_2 ≡ 460 μg. P. In view of the large blanks the agreement is most satisfactory.

Table 6. *Hydrolysis of Cori ester (glucose-1-phosphate) by a suspension of rabbit intestinal mucosa*

(Volumes made up tp 3·3 ml. with 0·2 M-acetate buffer pH 5·6. Temp. = 39°.)

Components of system	Manometer no. 1	2	3
1 ml. mucosa	+	−	+
300 μg. notatin + 1 ml. catalase	+	+	−
1 ml. Cori ester (0·1 M)	+	+	−
O_2 uptake in 85 min. (μl.)	240	39	35
Inorganic P at 85 min. (μg.)	1298	363	493

The initial very rapid O_2 uptakes in manometer 1 in each set of experiments is due to the accumulation of glucose during the interval required for equilibration. A more exact study of the kinetics of the reactions would require that either the phosphatase preparation or the ester be added from the dangling tube (see Introduction). The control experiment phosphatase + notatin indicated that the former contained no appreciable amount of glucose.

Activity of glucosidases

(1) *Emulsin* catalyzes the hydrolysis of amygdalin or mandelonitrile gentiobioside, the reaction proceeding as follows:

$$C_6H_5C(OH)CN.C_{12}H_{21}O_{10} + 2H_2O = C_6H_5CH(OH)CN + \underset{\text{glucose}}{2C_6H_{12}O_6},$$

$$C_6H_5CH(OH)CN = C_6H_5CHO + HCN.$$

The first stage is usually determined in a polarimeter, but is more readily followed with the use of notatin in the manner already described. The HCN produced in the second stage will, however, inactivate the catalase which is an essential component of the notatin system if a quantitative measure of glucose production is required. The most convenient way to overcome this difficulty is to add methaemoglobin which has a high affinity for HCN and which forms a stable CN-compound (Keilin & Hartree, 1936*b*) so protecting the catalase from inhibition. By this method curves 1 and 2 of Fig. 6 were obtained with 5 and 2·5 mg. emulsin respectively at 22° and pH 5·6. Each right-hand manometer flask received 2 ml. 10% crystalline methaemoglobin solution (for preparation see Keilin & Hartree, 1935) in addition to emulsin, amygdalin, notatin and catalase, and after taking readings for 45 min. the reaction mixtures were examined with a microspectroscope. At acid pH methaemoglobin shows four absorption bands in the visible region, the most prominent band being in the red at 632 mμ., while its CN′ derivative shows one broad band in the green. In both cases it was readily seen that only part of the methaemoglobin was combined with HCN. One molecule of methaemoglobin (mol. wt. 68,000) contains 4 Fe atoms and will combine with 4 molecules of HCN. The glucose produced in Exp. 1 (Fig. 6) can be determined from the O_2 uptake, and if it is assumed that the two phases of hydrolysis of amygdalin take place simultaneously then 2 molecules of glucose will appear for each molecule of HCN. On these grounds it can be calculated that if the O_2 uptake is 400 μl., the HCN produced will be sufficient to combine with 300 mg. methaemoglobin, i.e. 3 ml. 10 % solution. Since in Exp. 1 the HCN liberated is less than that required to react with 2 ml. 10 % solution, it follows that the second phase of the hydrolysis is slower than the first. The second phase can best be followed by the microspectroscopic technique already described by us using a double-wedge trough, and periodically matching the absorption spectrum of the reaction mixture with that of optical mixtures of methaemoglobin, and its CN′ derivative contained separately in the two compartments of the double-wedge trough (Keilin & Hartree, 1946).

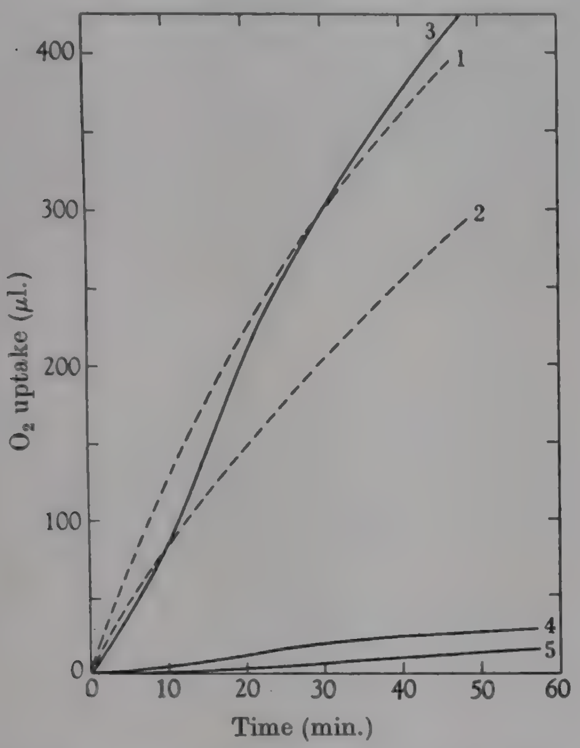

Fig. 6. (*a*) Hydrolysis of amygdalin by emulsin followed in terms of the oxidation of liberated glucose by notatin. Curve 1: 5 mg. emulsin, 60 mg. amygdalin, 2 ml. 10% methaemoglobin, 300 μg. notatin, 0·1 ml. catalase. 0·1 M-acetate buffer. Temp. = 22°. Curve 2: as 1 with 2·5 mg. emulsin. (*b*) Hydrolysis of sinigrin by sinigrinase. Glucose production followed by O_2 uptake in presence of notatin. Curve 3: 1 ml. sinigrin, 1 ml. sinigrinase, 0·1 ml. catalase, 300 μg. notatin, 0·1 M-phosphate buffer pH 5·6. Temp. = 22°. Curve 4: as 3 without notatin. Curve 5: as 3 without sinigrinase.

(2) *Emulsin* also catalyzes the hydrolysis of salicin to saligenin and glucose, and the reaction can be followed in the same way although here the methaemoglobin can be omitted.

(3) *Sinigrinase* was characterized by Neuberg & Wagner (1926) as the enzyme hydrolyzing sinigrin, a glucoside present in horse-radish root and mustard seed, the reaction proceeding as follows:

$$C_3H_5NC(OSO_3K)S\,.\,C_6H_{11}O_5 + H_2O$$
$$= \underset{\text{allyl mustard oil}}{C_3H_5NCS} + C_6H_{12}O_6 + KHSO_4.$$

The usual manometric technique with notatin is well suited to the study of the kinetics of this system. Crude sinigrin was prepared by boiling 100 g. crushed white mustard seed with 300 ml. H_2O for 15 min. and filtering with the aid of Hyflo Supercel (Johns-Manville, London). The clear, deep-yellow filtrate was not further purified. Sinigrinase was obtained by the method of Neuberg & Wagner from the mustard seed and its reaction with sinigrin in presence of notatin is shown in Fig. 6 (curves 3, 4, 5).

SUMMARY

1. Glucose oxidase or notatin which catalyzes the aerobic oxidation of glucose to gluconic acid, shows a marked specificity and a high affinity for glucose.

2. The stoicheiometric relationship between the glucose oxidized and the oxygen utilized makes possible an accurate manometric estimation of glucose in biological fluids containing other carbohydrates and proteins.

3. The kinetics of different enzyme reactions which yield glucose can be very easily studied manometrically by means of notatin. In this way a continuous record of the hydrolytic process can be obtained in terms of O_2 uptake.

4. The use of notatin has been applied to the manometric study of the reactions catalyzed by invertase *in vitro* and *in vivo*, maltase, salivary amylase, phosphatase, emulsin and sinigrinase.

5. The use of notatin provides a method for the quantitative study of the liberation of glucose from starch and glycogen by α-amylase. It has made possible the direct identification of glucose in such reactions.

6. By means of notatin it can be determined manometrically that living yeast cells (*T. utilis*) invert sucrose 500 times faster than is necessary to provide the monosaccharides necessary for normal respiratory requirements.

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Filter-paper Partition Chromatography of Sugars

1. GENERAL DESCRIPTION AND APPLICATION TO THE QUALITATIVE ANALYSIS OF SUGARS IN APPLE JUICE, EGG WHITE AND FOETAL BLOOD OF SHEEP

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The development by Consden, Gordon & Martin (1944) of a partition method of chromatography, in which sheets or strips of filter paper are used instead of the familiar packed column, has already shown its usefulness as a method for the qualitative analysis of protein or peptide hydrolysates for amino-acids, especially in circumstances where the amount of material available is small. Although the method is not suitable for obtaining accurate quantitative results, it has the special advantage that use can be made of very small differences in partition coefficient, with the result that useful separations may be obtained with as many as nineteen or twenty amino-acids on the same chromatogram. The present communication describes an extension of the method to the qualitative analysis of sugars, either in biological fluids or in the hydrolysis products of polysaccharides. Preliminary results have already appeared (Partridge, 1946).

The essentials of the method are as follows: A small quantity of the solution to be analyzed is introduced in the form of a circular spot near the top of a sheet or strip of filter paper. The paper is then hung vertically from a trough containing a solvent which has previously been saturated with water, so that the top edge of the paper is immersed in the solvent. The trough and the filter-paper strips are then suspended inside a closed vessel, the atmosphere in which is kept in a condition of saturation both with respect to water and to the solvent. A sharp horizontal liquid front forms, which advances down the paper at a speed dependent upon the physical properties of the solvent used. To irrigate a paper strip some 35 cm. long, 18–24 hr. are usually sufficient, after which the strip may be dried and the new positions of the components of the solution rendered visible by spraying the paper with an appropriate reagent.

Under these conditions, partition of the solute takes place between water bound by the cellulose and the solvent which is moving over the surface of the cellulose fibres. The distance moved by any solute in the direction of flow is then a function of (1) the partition coefficient of the solute between water and the wet solvent, (2) the volume of water bound by unit area of the filter paper, and (3) the volume of wet solvent held by unit area of the filter paper after irrigation. In any single experiment, factors (2) and (3) are substantially constant provided the filter paper is uniform in texture, thickness and water content, so that under ideal conditions the relative distance (R_F) moved by each solute is dependent upon its partition coefficient only.

The theory of the method has been dealt with in some detail by Consden *et al.* (1944), who give the definition

$$R_F = \frac{\text{distance moved by solute}}{\text{distance moved by advancing front of liquid}}.$$

These authors have shown experimentally that the values of R_F given by amino-acids on cellulose may be correlated fairly accurately with their partition coefficients as measured by direct methods, and the conclusion is drawn that true adsorption by the cellulose does not play a significant part.

In the work described below the reagent used to reveal the position of the spots formed by the reducing sugars has been almost exclusively a solution containing silver nitrate and a large excess of ammonia. This was sprayed on to the chromatogram after removal of the solvent used to irrigate it, and the filter-paper strip was then heated. In this way metallic silver was precipitated in the region occupied by reducing sugars, giving rise to brown or black spots. The $AgNO_3$-ammonia reagent has the advantage of being effective for all reducing sugars, and is therefore of general application to these, but for the non-reducing disaccharides or trisaccharides, other methods must be employed. For those containing a ketose, an acidic spraying reagent based on naphthoresorcinol is suitable, and details of its use are given in the text. In specialized cases other reagents were found to be of advantage, a particular instance being the use of *p*-dimethylaminobenzaldehyde as a reagent for the *N*-acetylhexosamines.

EXPERIMENTAL

With certain minor modifications, the experimental conditions that allow of separation of the amino-acids are also suitable for the qualitative analysis of sugars. Whatman no. 1 filter paper has been used exclusively, and for preparing strip chromatograms, the paper was cut into pieces 43 cm. long × about 12 cm. wide. The sugar solutions used were roughly 1% (w/v) with respect to each individual sugar, and 3–4 μl. containing 30–40 μg. of each sugar was introduced as a circular spot on a horizontal line ruled 7·5 cm. from the top of the paper strip. When a number of different sugar solutions was run on the same strip of paper, the spots were introduced at intervals of 1·5 cm. along the horizontal line. The strips were usually irrigated with the solvent for 18 hr. overnight. The solvent was dried off in an oven at 100–105°, the position of the solvent boundary being marked in ink before transfer to the oven. In order to reveal the position of the sugars the paper was sprayed rapidly and evenly with a mixture containing equal parts of silver nitrate solution (0·1 N) and ammonia solution (5 N), and the strip replaced in the oven (105°) for 5–10 min.; the sugars appeared as dark brown spots on a white or light brown ground. In runs carried out in phenol the lower part of the paper was usually uniformly coloured light brown, the upper part being white or light grey. The developed strips began to darken owing to the presence of silver salts a few hours after they had been removed from the oven, but if a permanent record was desired, the strips were washed first in distilled water and afterwards in running tap water for about 1 hr., followed by drying.

The apparatus used was that described by Consden *et al.* (1944). It was found, however, that the glass troughs could be replaced with advantage by troughs of a similar pattern made from stainless steel. For drying off the solvent and heating the papers a special oven was constructed. This had a large glass window in the front in order to allow the development of the spots to be kept under observation as the temperature increased after introducing a sprayed chromatogram. This feature was of value, since when using $AgNO_3$-ammonia as a spraying reagent it was found that certain sugars (such as glucosamine and chondrosamine) react at lower temperatures, and this effect often served as a useful confirmation of an identification based on R_F value. The oven was equipped with a small centrifugal fan which caused air heated by a bank of electrical strip heaters to be drawn slowly through the drying chamber and then to be discharged outside the building.

Use of naphthoresorcinol as a reagent for di- and trisaccharides containing ketoses

For many non-reducing oligosaccharides or glycosides the use of an acidic reagent is necessary in order to secure hydrolysis. It was found that after spraying a filter-paper chromatogram with a dilute solution of an acid and heating to 80–100°, concentration of the acid took place to a point at which dehydration of the sugars occurred, with consequent liberation of substituted furfurals. The position of the sugars could therefore be revealed by making use of the well known colour reactions of furfurals with phenolic reagents. Under certain conditions the reaction was found to be highly specific for the ketoses. A number of phenolic substances were tested for suitability as spraying reagents, and of those examined naphthoresorcinol appeared most satisfactory, although other common substances of this type, such as α-naphthol or resorcinol, were also effective. The reaction took place with phenol itself, and in chromatograms irrigated with this solvent there was usually sufficient phenol left on the paper to develop a colour with fructose on spraying with an acid and heating in the oven. Collidine inhibited the reaction to some extent, and this solvent was avoided where phenolic reagents were to be used.

A spraying reagent based on hydrochloric acid (0·25 N) and containing naphthoresorcinol (0·1% w/v) was found to be effective in producing strong red colours with the ketoses at 100–105°, but since the mineral acid tended to break down the cellulose of the filter paper it was found preferable to replace it by trichloroacetic acid. The reagent finally adopted was as follows: *Solution* (*a*) naphthoresorcinol, 0·2% (w/v) in ethanol; *solution* (*b*) trichloroacetic acid, 2·0% (w/v) in water. Equal volumes of the two solutions were mixed immediately before use, and the paper sprayed evenly with the mixture. The paper was then allowed to dry off partially at room temperature before being introduced into the oven at 100–105° for 5–10 min. Fructose, sorbose, sucrose and raffinose gave very strong red spots, the colour of which was stable for at least 12 hr.

Under the conditions described, the reaction was very selective for the ketoses, other sugars giving no more than a trace of colour at temperatures above 100°. However, on allowing the heated chromatograms to stand for a few hours in the open air, the pentoses and uronic acids developed strong blue colorations. The presence of moisture appeared to be necessary for the development of the blue colour, and in cases where it was desired to reveal the positions of the pentoses and hexuronic acids the sprayed chromatogram was heated in a humid atmosphere at 70–80°. For this purpose, open baths of water were inserted in the oven above the heaters. At 70–80° in humid air, development of the deep blue or purple colours required 10–15 min., and under these conditions the colour due to the ketoses was orange-brown. The blue or purple colour was very stable, and was particularly intense in the case of the hexuronic acids.

Use of the Morgan & Elson reaction for hexosamine

The reagents devised by Elson & Morgan (1933) and Morgan & Elson (1934) for the colorimetric estimation of hexosamines and *N*-acetylhexosamines were adapted for use with the filter-paper technique as a second method of revealing the presence of the amino sugars. The method is of particular value for *N*-acetylglucosamine, since this substance is only slowly oxidized by the ammoniacal silver nitrate reagent, but gives an excellent reaction with *p*-dimethylaminobenzaldehyde.

The reagents were as follows:

(*a*) *Acetylacetone reagent.* Solution (1): Acetylacetone (0·5 ml.) dissolved in butanol (50 ml.). Solution (2): 50% (w/v) aqueous KOH (5 ml.) and ethanol (20 ml.). 0·5 ml. of solution (2) were added to 10 ml. of solution (1) just before the reagent was required. Crystals occasionally appeared in the mixed solution, but they readily redissolved on addition of a few drops of 50% (v/v) aqueous ethanol. The reagent was not stable and was made up daily from fresh acetylacetone.

(*b*) *p-Dimethylaminobenzaldehyde reagent.* *p*-Dimethylaminobenzaldehyde (1 g.) after recrystallization from aqueous ethanol was dissolved in ethanol (30 ml.) and 30 ml. of conc. HCl added. The solution was then diluted with 180 ml. of redistilled butanol. The reagent was stable in ethanol-HCl solution and a stock solution in the solvent could be stored for several weeks.

After evaporating off the solvent, the chromatograms were sprayed with solution (*a*) and heated in the oven for 5 min. at 105°. The dry paper strips were then sprayed with solution (*b*) and returned to the oven for a further short heat treatment (5 min.) at 90°. Under these conditions the free hexosamines gave cherry-red colorations which were stable for several days. *N*-Acetylglucosamine gave a strong purple-violet colour which was also stable. However, unlike the free hexosamines, the *N*-acetyl derivatives gave strong violet colorations with the *p*-dimethylaminobenzaldehyde reagent alone, without any previous treatment with acetylacetone, and this reaction could be utilized to serve as a confirmatory test for the presence of the *N*-acetylated derivatives.

Other reducing sugars also gave colours with these reagents under the conditions described above, but the colorations were usually very light and faded rapidly, particularly on raising the oven temperature. The colours given by various neutral reducing sugars ranged from blue to pink-violet, each sugar giving a characteristic shade, but it was considered doubtful if the method would be of value for revealing the presence of the neutral sugars since the reaction was not sufficiently sensitive.

Solvents

Phenol. It was found convenient to prepare a stock solution containing phenol (detached crystals, B.P.) (900 g.) and water (100 g.). This mixture remained liquid at room temperature, and to prepare a saturated solution, the stock solution was gently shaken with an excess of water, and after separation the lower phenol layer was run off into the trough of the chromatographic chamber. The aqueous layer was run into the bottom of the chamber in order to maintain saturated conditions in the air above it. Some batches of phenol were not suitable for use as purchased, since they gave rise to a heavy black discoloration with the $AgNO_3$-ammonia reagent. Such samples could be improved considerably by steam distillation, but in one case this treatment was insufficient and the sample was further treated by shaking at intervals for several days in a large bottle with 1% (w/v) ammonia solution until the mixture acquired a deep blue colour. After steam distillation the solvent was then found to be satisfactory.

When amino sugars were under investigation, it was necessary to carry out irrigation with phenol in an atmosphere containing ammonia, since otherwise these basic sugars gave rise to elliptical spots or trails. For this purpose, the aqueous layer in the bottom of the chamber was brought to a known concentration with NH_3 solution. However, when ammonia was used a few crystals of KCN were also added to the aqueous liquid, since catalytic oxidation processes take place under the influence of traces of copper (Consden *et al.* 1944), and, in the absence of a small concentration of HCN, the advancing phenol front on the paper becomes deeply stained with dark-coloured oxidation products.

s-Collidine. This solvent was purchased as a dark-coloured liquid. Bromine (5–10 ml.) was cautiously added to the solvent (1 l.) in a large unstoppered flask with frequent vigorous shaking; after removal of the precipitate by filtration crushed sodium thiosulphate was added with further shaking, and the liquid was again filtered. The solvent was then allowed to stand 24 hr. over solid NaOH and after filtration was distilled, a middle fraction boiling at 172° being retained. Prepared in this way, the liquid remained colourless for several weeks, and after saturation with water at room temperature was used for developing the chromatograms without further additions. (This method of purification was communicated privately by Dr A. J. P. Martin.)

n-Butanol-acetic acid mixture. The butanol was purified by distillation and the dry solvent (40 ml.) was mixed with glacial acetic acid (10 ml.) and water (50 ml.). After shaking the mixture, the aqueous layer was run into the bottom of a dry chamber, and the solvent layer used to fill the trough. Since the mixture is a three-component system the volume of water contained in it is important.

RESULTS

Table 1 shows the R_F values obtained with a number of neutral sugars in certain of the more suitable solvents and solvent mixtures. In general, it has been found that only those solvents that give rise to two phases with water lead to useful separations, and further that R_F values are generally higher the

Table 1. *R_F values of neutral sugars in various solvents on Whatman no. 1 paper (corrected to 20°)*

Solvent ...	Phenol	*s*-Collidine	*n*-Butanol, 40%* Acetic acid, 10% Water, 50%	*n*-Butanol, 45%* Ethanol, 5% Water, 49%	*n*-Butanol	*iso*Butyric acid	Methyl ethyl ketone
Additions	NH_3 (1% w/v), HCN	None	None	NH_3 (1% w/v)	NH_3 (1% w/v)	None	NH_3 (1% w/v)
D-Glucose	0·39	0·39	0·18	0·105	0·070	0·13	0·025
D-Galactose	0·44	0·34	0·16	0·090	0·060	0·14	0·015
D-Mannose	0·45	0·46	0·20	0·130	0·100	0·15	0·050
L-Sorbose	0·42	0·40	0·20	0·120	0·085	0·16	0·050
D-Fructose	0·51	0·42	0·23	0·135	0·100	0·18	0·045
D-Xylose	0·44	0·50	0·28	0·170	0·125	0·19	0·090
D-Arabinose	0·54	0·43	0·21	0·145	0·100	0·21	0·075
D-Ribose	0·59	0·56	0·31	0·210	0·180	0·22	0·165
L-Rhamnose	0·59	0·59	0·37	0·285	0·220	0·30	0·180
D-Deoxyribose	0·73	0·60	—	—	—	0·32	—
L-Fucose	0·63	0·44	0·27	—	—	—	0·095
Lactose	0·38	0·24	0·09	0·0	0·0	0·070	0·0
Maltose	0·36	0·32	0·11	0·15	0·01	0·085	0·0
Sucrose	0·39	0·40	0·14	—	—	—	—
Raffinose	0·27	0·20	0·05	—	—	—	—

* All percentages are v/v.

greater the water content of the solvent phase. Since the amount of water carried by the solvent phase is dependent upon temperature, mobilities are higher the nearer the temperature of the chamber approaches the critical solution temperature of the solvent. Thus when phenol is used, an increase in temperature gives rise to increasing R_F values, while in *s*-collidine the effect is reversed. The temperature effect becomes less marked at working temperatures well below the critical point, and when phenol or collidine is used normal variations in the temperature of the room during a run can be tolerated. It is, however, advisable to lag the chamber in order to reduce such temperature disturbances to a minimum.

Since the differences in temperature between summer and winter working affect the R_F values of all the sugars to approximately the same degree, a standard sugar (glucose) was included in every chromatogram, and all R_F values obtained at temperature $t°$ were then multiplied by a factor a_t, where

$$a_t = \frac{R_F \text{ value of glucose at } 20°}{R_F \text{ value of glucose at } t°}.$$

Applying this correction it has been found that R_F values obtained on the same batch of filter paper are remarkably constant in spite of the uncontrollable variations in other factors met with in day-to-day working.

The behaviour of a solvent may be influenced by the addition to it of a small quantity of a solvent more readily soluble in water. This increases the solubility of water in the new solvent phase, and thus effects a general increase in R_F values. Thus the addition of acetic acid to *n*-butanol leads to an increase in R_F values and, in this case, to an improvement in the separations obtained with some of the sugars.

The behaviour of sugar mixtures

The R_F value given by a neutral sugar is not substantially affected by the presence of other sugars at comparable concentration in the same solution, although small deviations have sometimes been noted in the chromatograms given by solutions containing four or five different sugars. Pl. 2 (*a*) shows a 40 hr. chromatogram obtained in collidine with a solution containing ribose, mannose, fructose, galactose, lactose and galacturonic acid, the concentrations being 1% (w/v) with respect to each sugar. For the purpose of reference the individual sugars were run alongside the mixture. The photograph in Pl. 2 (*a*) should be compared with Fig. 1 (p. 242), which illustrates the degree of separation obtainable by use of two solvents, phenol-1% (w/v) NH_3 and collidine, and has been obtained by plotting the R_F values of the sugars in phenol as ordinates and the values in *s*-collidine as abscissae.

Sensitivity. Pl. 2 (*b*) shows a chromatogram obtained in collidine with a solution containing glucose (1% w/v) and xylose (1% w/v). The solution was applied in amounts varying from 4 µl. to 0·5 µl. The R_F values obtained by measuring the centre of the spots show no systematic variation with the amount of sugar present and this has been found to be the general rule. The amount of sugar applied (5–40 µg.) covers the range over which the method is useful for general purposes, but with some sugars (particularly glucose) much smaller amounts may be detected, and for special purposes considerably larger amounts of sugar have been applied without causing serious distortion of the spots.

Interfering substances. Substances other than sugars present in the test solution may interfere in a variety of ways, and for the purpose of clarity they may be classified into groups as follows:

(*a*) Reducing substances which react with the ammoniacal silver nitrate reagent.

(*b*) Substances which may have mobilities close to those of the sugars it is desired to identify and which may affect the R_F values of the sugars by 'salting out'.

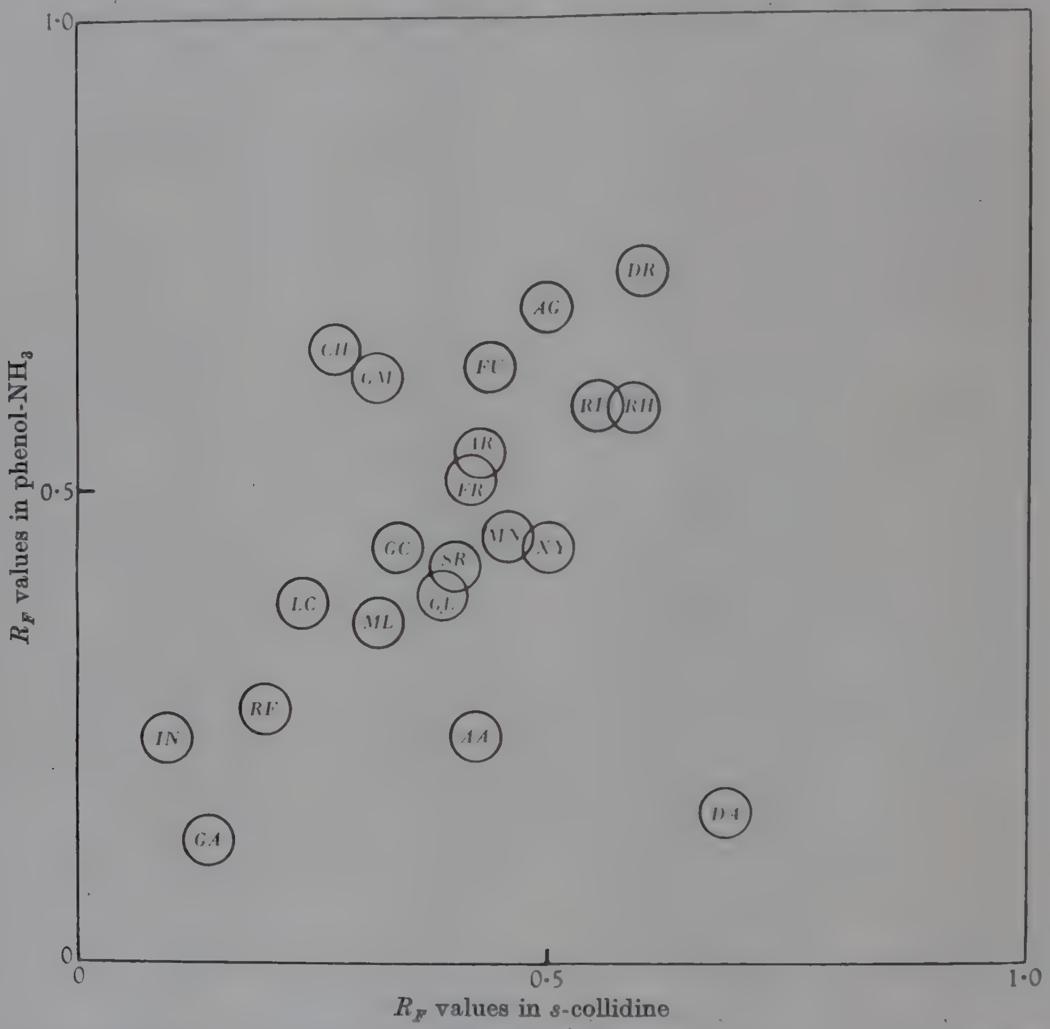


Fig. 1. Illustrating the separation obtainable by use of two solvents. The R_F values in phenol-1 % NH_3 and *s*-collidine are plotted at right angles. *AA*, ascorbic acid; *AG*, acetylglucosamine; *AR*, arabinose; *CH*, chondrosamine; *DA*, dehydroascorbic acid; *DR*, deoxyribose; *FR*, fructose; *FU*, fucose; *GA*, galacturonic acid; *GC*, galactose; *GL*, glucose; *GM*, glucosamine; *IN*, inositol; *LC*, lactose; *ML*, maltose; *MN*, mannose; *RF*, raffinose; *RH*, rhamnose; *RI*, ribose; *SR*, sorbose; *XY*, xylose.

(*c*) Inorganic salts, which in addition to causing effects as under (*b*), may also be involved in reactions of the ion-exchange type leading to the localization of strong acids or bases which give rise to silver-reacting spots by modification of the cellulose of the filter paper.

Numerous substances occurring in tissue extracts were tested for their reaction with $AgNO_3$-ammonia under the conditions used in developing the chromatograms. Purines, creatine, creatinine and lactic acid and the common amino-acids were inactive, but inositol gave a weak brown spot developing at 110°, or at a rather lower temperature if a small concentration of NaOH was added to the spraying reagent. Ascorbic acid, gallic acid and tannic acid gave an immediate black coloration in the cold, and could readily be distinguished from the sugars by this means.

Interfering effects of class (*b*) were noticed in attempts to identify reducing sugars occurring in complex biological mixtures such as fruit juices or muscle press juice. In chromatograms obtained with the untreated extracts it was not uncommon to find that the spot due to a reducing sugar was badly distorted, or displaced by substances which revealed themselves as white spots or trails on the cream or buff-coloured ground of the chromatograms. As described below, reliable results could be obtained with such extracts only if steps were taken to remove the electrolytes, preferably by means of ion-exchange materials. However, the presence of such substances as the amino-acids could usually be tolerated provided their concentrations were not much greater than those of the sugars under test; this was particularly so if the non-sugar substances had R_F values either above or below the range occupied by the sugars.

The action of the paper chromatogram in separating the ions of inorganic salts is described in more detail by Westall in the note attached to this communication. When sufficient of the test solution was available the effect could be eliminated by making use of ion-exchange materials, and, in fact, the preliminary removal of all but small concentrations of electrolytes proved to be a necessary step before commencing the analysis of any unknown solution. With the hydrolysate of a pure carbohydrate, where the mineral acid may be removed quantitatively, the presence of interfering substances was not to be expected, and, in fact, in the example given in the second part of this communication (this vol. p. 251), the analysis of such a carbohydrate was uncomplicated by interference effects.

Reducing sugars with acidic or basic properties

The behaviour of the neutral reducing sugars on the filter-paper chromatograms was apparently uncomplicated by the various tautomeric equilibria known to occur in their aqueous solutions, and the spots revealed by the $AgNO_3$-ammonia reagent showed no tendency to trail. However, a few of the more common sugar derivatives with basic or acidic properties were investigated, and with some of these substances the products of reversible reactions taking place at or near room temperature showed some separation on the chromatograms. The results obtained with the sugar acids and amino sugars are given below; R_F values in the more useful solvents are shown in Table 2.

Glucuronic acid and its lactone. The source of glucuronic acid was L-menthol-D-glucuronide prepared biosynthetically by the method of Williams (1939). A sample of crystalline glucurone, $[\alpha]_D + 19{\cdot}7$, m.p. 178–180°, was prepared from this substance by hydrolysis with $0{\cdot}2$ N-sulphuric acid according to the method of Quick (1927). The first hydrolysis product is a mixture containing glucuronic acid and its lactone in the proportion of 7 : 3, but crystallization from hot glacial acetic acid favours lactone formation and the product after two recrystallizations is the pure lactone.

The mixture of glucurone and glucuronic acid formed by first hydrolysis when applied to the filter-paper chromatogram gave rise to two well separated spots after development with the solvents (Pl. 3 (*a*) and (*b*)). The slower moving spot was due to glucuronic acid and was usually heart-shaped or elliptical, sometimes appearing with a marked forward trail. The spot due to glucurone had a high R_F value in all solvents and was invariably circular. The glucurone spot, however, developed more slowly than glucose after spraying with the silver reagent, and for this reason might easily be missed in the examination of the chromatograms.

Crystalline glucurone gave a single circular spot of high R_F in acidic solvents, but in basic solvents the spot was often accompanied by a backward trail, indicating conversion to glucuronic acid during the course of the run (Pl. 3 (*b*)). In the presence of ammonia vapour from a 1 % (w/v) aqueous ammonia solution, phenol behaves as a basic solvent, and the ammonia concentration is sufficient to complete the conversion of glucurone to the salt of glucuronic acid. Under these conditions the spot due to glucurone was entirely absent, the application of solutions of crystalline glucurone giving rise to the glucuronic acid spot accompanied by a forward trail.

Glucuronic acid reduced the $AgNO_3$-ammonia reagent very rapidly at 100°, but the lactone reduced only slowly at 110°. For this reason, when the lactone was suspected to be present, NaOH solution was added to the $AgNO_3$-ammonia reagent to bring the concentration of the NaOH to $0{\cdot}05$ N. Glucurone was thereby converted on the filter paper to the sodium salt of glucuronic acid which rapidly reduced the silver reagent. Development of the colour was carried out at 110°, but the paper could not be heated longer than 3–5 min. because of excessive darkening. Butanol-acetic acid mixture was found the most suitable solvent for mixtures containing glucurone; the silver reagent containing sodium hydroxide was not suitable for use with phenol as the chromatographic solvent.

Galacturonic acid. D-Galacturonic acid does not readily form a lactone, and the crystalline acid gave rise to a single spot of low mobility which, like that due to glucuronic acid, was usually heart-shaped and often showed a short forward trail. Glucuronic acid and galacturonic acid may be differentiated by the formation of the glucurone spot by the former. R_F values are given in Table 2.

Glucosamine and chondrosamine. In basic solvents (phenol-NH_3 and collidine) the hexosamine hydrochlorides travelled as well defined circular spots which rapidly reduced the silver reagent at low temperatures (90°). In neutral or acidic solvents, however (phenol, butyric acid, butanol-acetic acid), the hexosamines appeared after development as brown spots with black forward trails. The trails reacted with the silver reagent at low temperatures and appeared much more rapidly than the spots during development. It appears, therefore, that the hexosamines travel as the free base under alkaline conditions, but in neutral or slightly acidic solvents give rise to equilibria between free base and cation which result in partial separation, the free base travelling faster than the ion.

The Morgan & Elson reagents described above may be used to show the position of the spots due to the hexosamines, and in cases where the hexosamine spot is in close proximity to that due to another sugar, the specific reaction for the former is of considerable value.

N-Acetylglucosamine. This substance had a high mobility in most solvents, but gave a rather weak reaction with the ammoniacal silver nitrate reagent. However, the reaction with *p*-dimethylaminobenzaldehyde described above was very sensitive and also specific for the *N*-acetylhexosamines, and could be used with advantage wherever the *N*-acetyl derivatives were suspected.

Ascorbic acid. Freshly prepared aqueous solutions (1 % w/v) of synthetic L-ascorbic acid were used. In phenol-ammonia, collidine and *n*-butanol-acetic acid, ascorbic acid gave well characterized spots which produced an immediate black colour on spraying with the $AgNO_3$-ammonia reagent in the cold. The presence of HCN was necessary when using phenol-ammonia as the chromatographic solvent in order to suppress catalytic oxidation of the ascorbic acid. The values for R_F found are given in Table 2.

Table 2. R_F *values of a number of substances related to the sugars on Whatman no.* 1 *paper* (*corrected to* 20°)

Solvent	Phenol	*s*-Collidine	*n*-Butanol, 40 %* Acetic acid, 10 % Water, 50 %	*iso*Butyric acid
Additions	1 % NH_3, HCN	None	None	None
D-Galacturonic acid	0·13	0·14	0·14	0·09
D-Glucuronic acid	0·12	0·16 [0·72]†	0·12 [0·32]†	0·08 [0·22]†
D-Glucurone	0·12	— [0·72]†	— [0·33]†	— [0·22]†
D-Glucosamine hydrochloride	0·62	0·32	0·13 [0·17]‡	0·05 [0·20]‡
Chondrosamine hydrochloride	0·65	0·28	0·12 [0·16]‡	— [0·19]‡
N-Acetylglucosamine	0·69	0·50	0·26	0·25
L-Ascorbic acid	0·24	0·42	0·38	0·19
Dehydroascorbic acid	0·16	0·68	0·27	0·16
i-Inositol	0·23	0·10	0·09	—

* All percentages are v/v.
† Values in brackets [ ] due to the lactone.
‡ Values in brackets [ ] due to free base; the two spots were connected by a trail.

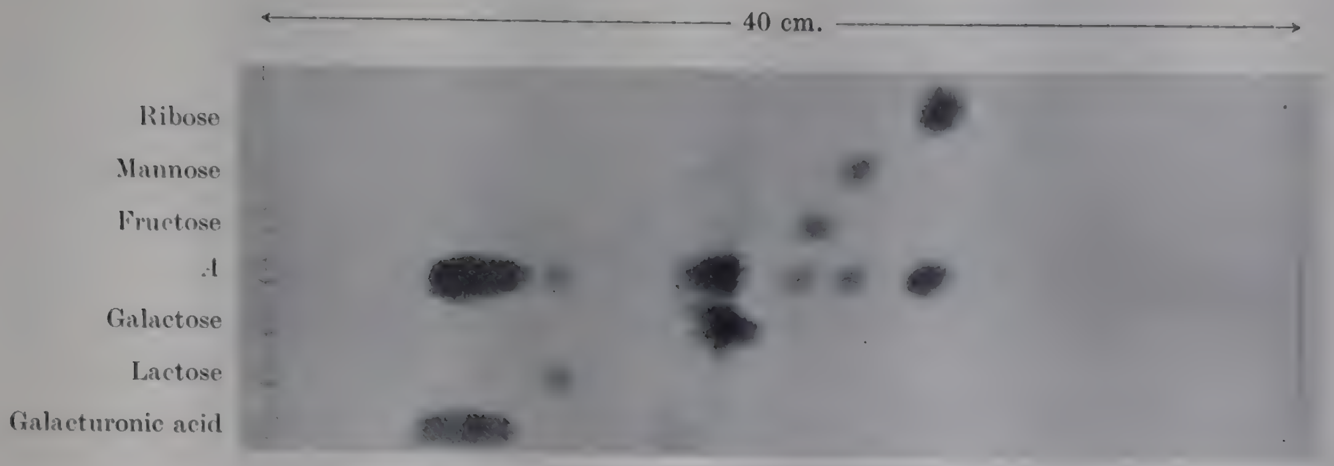
Dehydroascorbic acid. Dehydroascorbic acid was prepared by oxidation of ascorbic acid with iodine according to the methods given by Herbert, Hirst, Percival, Reynolds & Smith (1933).

(*a*) An aqueous solution of ascorbic acid was oxidized with the calculated amount of iodine dissolved in ethanol, the addition being made at room temperature.

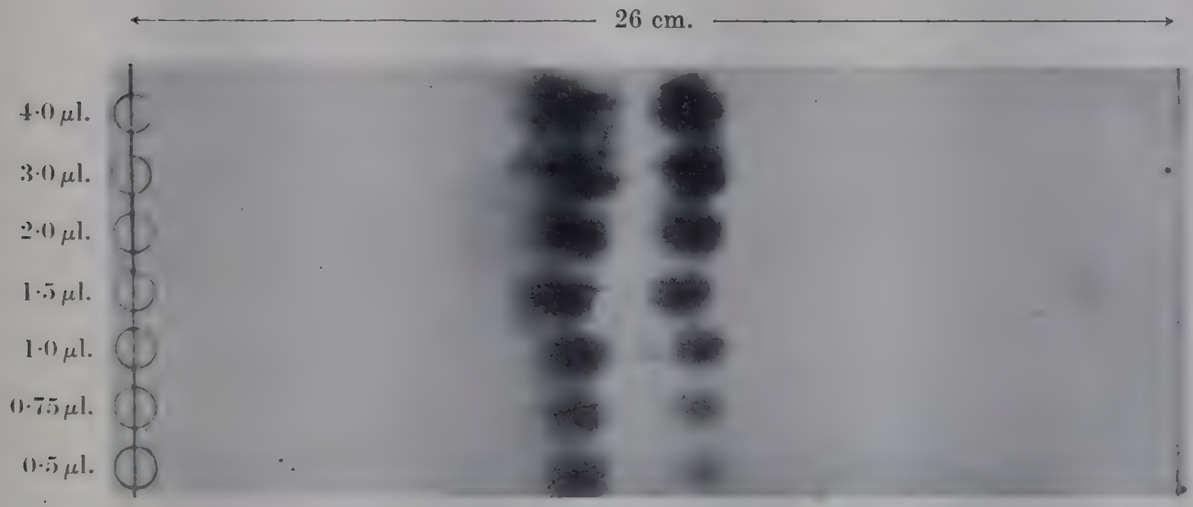
(*b*) An aqueous solution of ascorbic acid (10 ml. of 0·2 M) was titrated with 0·2 N-iodine in aqueous KI solution until the end point was reached. The solution had $[\alpha]_D + 53°$ (l, 2·0) (calculated on the weight of ascorbic acid taken) 30 min. after preparation. The solution was then titrated with N-NaOH to the temporary end point (phenolphthalein) in order to neutralize the mineral acid formed during the oxidation but to avoid salt formation with dehydroascorbic acid and consequent rupture of the lactone ring.

(*c*) A neutralized solution of dehydroascorbic lactone was prepared as in method (*b*), but the addition of N-NaOH was carried past the temporary end point until a permanent colour with phenolphthalein was obtained. The addition was carried out at room temperature. The solution contained the sodium salt of dehydroascorbic acid and was yellow in colour.

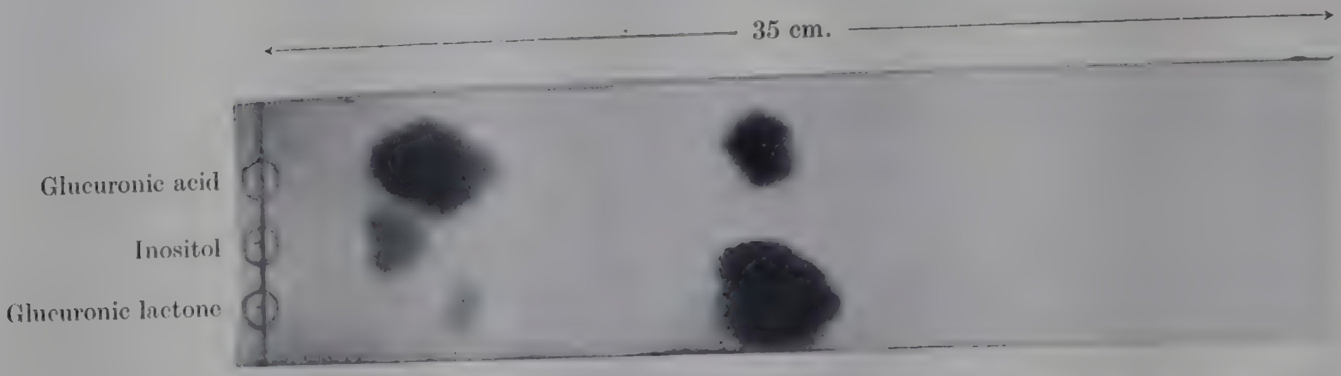
When freshly prepared the solutions (*a* or *b*) containing the lactone and that (*b*) containing the sodium salt of the free acid gave the same chromatogram, all three solutions yielding a single purple-brown spot which developed rapidly with the $AgNO_3$-ammonia reagent at 80–90°. This result was unexpected since glucuronic acid-glucurone mixtures gave rise to two distinct spots, widely separated in R_F value. However, inspection of Table 2 shows that the R_F values of dehydroascorbic acid in different solvents are markedly anomalous, since mobility is very high in collidine, but low in phenol-NH_3, and it is possible that the components of the system are too rapidly interconvertible to allow of the complete separation of either during the course of the run. In such a system the R_F value of the mixture would be expected to lie near that of the predominant component, and would therefore be markedly affected by any alteration in the solvent likely to disturb the equilibrium between the two components either in the solvent phase or in the water phase. The spots due to dehydroascorbic acid were usually


40 cm.
Ribose
Mannose
Fructose
A
Galactose
Lactose
Galacturonic acid

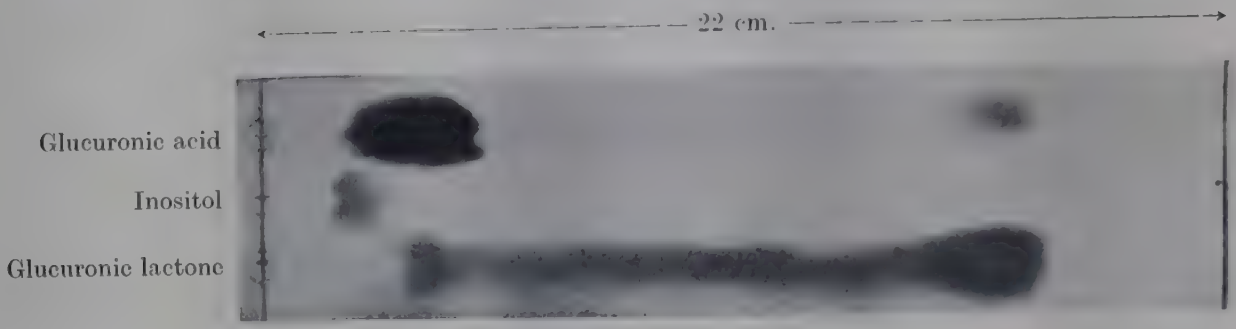
(*a*) Filter-paper chromatogram of a mixture containing six sugars. The mixture was introduced in the circle marked '*A*' and the individual sugars were placed in the other circles as indicated. The chromatogram was run 40 hr. in *s*-collidine.


26 cm.
4·0 μl.
3·0 μl.
2·0 μl.
1·5 μl.
1·0 μl.
0·75 μl.
0·5 μl.

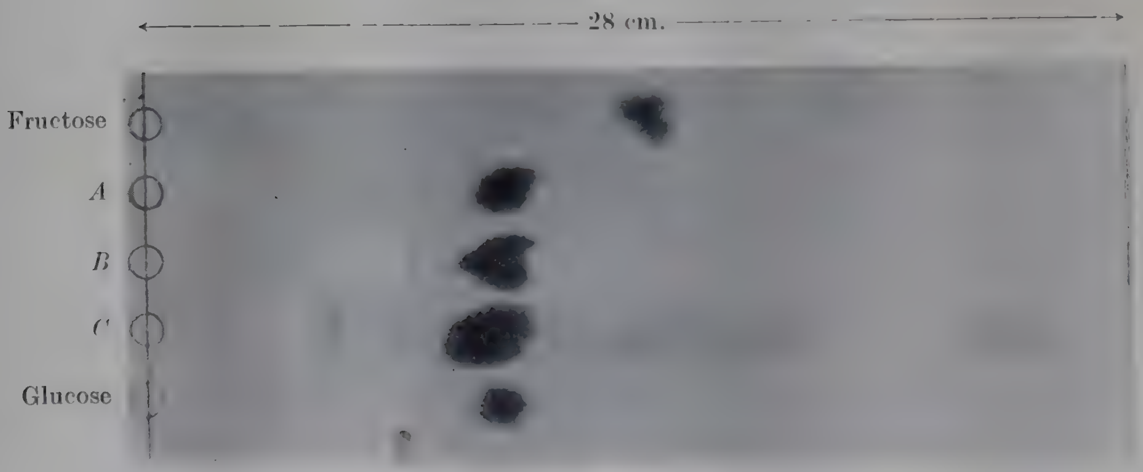
(*b*) Filter-paper chromatogram of a solution containing glucose (1 % w/v) and xylose (1 % w/v). The volume of solution applied to each circle is indicated. The chromatogram was run 20 hr. in *s*-collidine.


35 cm.
Glucuronic acid
Inositol
Glucuronic lactone

(*a*) Chromatogram run in butanol-acetic acid mixture. The chromatogram was sprayed with ammoniacal silver nitrate solution containing NaOH to a concentration of 0·05 N.


22 cm.
Glucuronic acid
Inositol
Glucuronic lactone

(*b*) Chromatogram run in *s*-collidine and sprayed with ammoniacal silver nitrate solution containing NaOH to a concentration of 0·05 N.


28 cm.
Fructose
A
B
C
Glucose

(*c*) Chromatogram of an extract of egg white in aqueous ethanol. *A*, extract treated with 'Zeo Karb 215' and 'Deacidite'. *B*, extract treated with 'Zeo Karb' only. *C*, untreated extract. The chromatogram was run 18 hr. in phenol-1 % NH_3.

elliptical and showed marked forward trailing in collidine, but they were well separated from those due to ascorbic acid in most solvents.

Solutions (*a*) and (*c*) were unstable, and after keeping for 24 hr. gave traces of a second reducing substance yielding a slow-moving spot in collidine and butanol-acetic acid. This spot gave a brown colour with the silver reagent, but required heating to 100–110° for colour development. Solution (*a*) regenerated ascorbic acid on keeping for a few days at $+2^\circ$, the solution giving rise to a third, small, but well defined spot which oxidized the silver reagent in the cold, and had an R_F value identical with that of ascorbic acid.

Inositol. The sample of *i*-inositol gave no reduction with alkaline ferricyanide (Hagedorn & Jensen, 1923) and was nitrogen-free. It was observed, however, that the substance gave a slow reduction with the $AgNO_3$-ammonia reagent under the ordinary conditions used in developing the paper chromatograms. The rate at which reduction took place was increased when NaOH was added to the $AgNO_3$-ammonia reagent to a concentration of 0·05 N, but even under these conditions the brown spot due to inositol was much less intense than that due to an equivalent concentration of a reducing sugar.

The mobility of inositol was low in all solvents (R_F values are given in Table 2), and consequently it was readily distinguished from aldoses and ketoses; but since the colour obtained with the silver reagent was not strong it was easily missed in the examination of chromatograms, particularly if present in the test solution in low concentration.

Application of method to extracts of biological origin

The object of this investigation was to establish a method for the qualitative analysis of polysaccharide hydrolysates. In such liquids the problem is relatively simple, since once the free mineral acid has been removed the solution is likely to be relatively free from salts, organic acids and other substances which might interfere. It was, however, considered of interest to explore the chromatographic analysis of a few examples of extracts of biological origin known to contain reducing sugars. The results of three of these investigations are given below.

Detection of glucose, fructose and sucrose in apple juice. An apple-juice extract was prepared from Bramley's Seedling apples and ethanol was added to a concentration of 80 % (v/v). After standing a few days at $+2^\circ$, the solution was filtered, and the clear supernatant (500 ml.) retained for the analytical experiments. Standard methods of analysis showed: fructose, 0·34 %; glucose, 0·25 %; sucrose, 0·07 %; malic acid, 0·40 %; total dry solids, 2·45 %.

Part of the ethanolic solution was evaporated to small bulk under reduced pressure and made up with distilled water so that the resulting concentration was five times the original. This solution was clarified by centrifugation and used for the chromatographic experiments. In the description of the results which follows, the runs are classified according to the solvent used. This was usually neutral or alkaline phenol, since the best separation between glucose and fructose is obtained with phenol as the solvent.

(*a*) *Solvent, phenol; additions, none.* Brown spots of correct mobility for glucose and fructose could be distinguished readily, but they were much distorted in shape by the presence of other material of similar R_F value. Among the interfering substances observed were two well defined spots due to pigments of R_F 0·59 and 0·49, which gave transitory green or brown colours in the cold on spraying with $AgNO_3$-ammonia. In addition to the pigments there were a number of substances of low mobility which gave an immediate black colour in the cold on spraying with the $AgNO_3$-ammonia reagent. These were afterward shown to be tannins. There were further substances giving spots or trails which appeared colourless against the pale grey or cream colour of the paper background.

(*b*) *Solvent, phenol; additions,* NH_3 1 % (*w/v*), KCN. The appearance of the chromatograms was much improved in this solvent. Most of the acidic interfering substances had now very low mobilities due to the presence of the ammonia, and therefore did not interfere with the development of the spots due to the reducing sugars. Glucose and fructose gave well defined spots of correct R_F value, and the black colour due to the tannins was confined to a short trail springing from the position originally occupied by the test solution. However, since it was desired to test for the presence of other reducing sugars possibly present in small amounts, further runs were carried out with higher concentrations of the test solution. These were rather unsatisfactory, due to the simultaneous increase in concentration of interfering substances. For this reason an attempt was made to utilize ion-exchange reagents to remove acids, bases and salts while leaving the non-electrolytes in solution.

Use of ion-exchange columns. 'Zeo-Karb 215'* was used for removal of bases and cations and 'De-acidite'* for removal of acids. A portion (100 ml.) of the 80 % ethanolic apple-juice extract was evaporated to small bulk, and made up again to its original volume with distilled water in readiness to pass through the columns. In order to minimize the possibility of adsorption of sugars by the ion-exchange resins, sufficient of the test solution was passed to saturate the deacidite column with respect to anions, and the first runnings from both columns were rejected.

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* Both these reagents may be obtained from Permutit Co. London.

The columns were prepared by taking sufficient of the wet ion-exchange material to make up a volume of 22 ml. This gave a column 16 cm. in height when the material was packed into the filtration tubes. The deacidite was activated with 2N-NaOH solution, followed by thorough washing, while 2N-HCl solution was used to activate the zeo-karb. The test solution was passed slowly through the zeo-karb column and immediately transferred to the deacidite column. The pH of the solution flowing from the second column was followed by means of indicators, and the experiment was stopped when the reaction fell to pH 6·5, indicating approaching saturation of the deacidite. In all, 90 ml. of the solution were passed through both columns in this way, the first 15 ml. run from either column being rejected.

A portion (50 ml.) of the effluent was concentrated to 5 ml., and the small precipitate which formed was removed by centrifugation. The concentrated effluent solutions were tested by the filter-paper technique using phenol-NH_3, butanol-acetic acid mixture and collidine as solvents. The developed papers indicated freedom from interfering substances, and the R_F values given by glucose and fructose in the concentrates were not detectably different from those given by authentic glucose and fructose solutions run on the same chromatogram.

In order to ascertain if adsorption of reducing sugars by the columns was likely to be a serious consideration in further applications of the technique, the experiment was repeated under identical conditions except that 10 ml. of the test solution instead of 90 ml. were passed down the columns, and no part of the effluent was rejected. The relative intensity of the glucose and fructose spots was unaffected in the resulting chromatograms, and from this it was concluded that for qualitative purposes the method was satisfactory as far as glucose and fructose were concerned.

Detection of sucrose. Chromatograms were set up with the concentrated effluent from the ion-exchange columns used for the detection of glucose and fructose. Phenol and butanol-acetic acid were used as solvents and fructose, glucose and sucrose were included as reference sugars. After removal of the solvent by evaporation the chromatograms were sprayed with the naphthoresorcinol-trichloroacetic acid reagent as described in the experimental section. The spots due to fructose and sucrose in the mixture gave strong red colorations and had R_F values identical with those of the reference sugars. Glucose gave no colour with this reagent.

Detection of glucose in the white of egg. The white was separated from a hen's egg taken a few hours after laying. After dilution with water to 100 ml., absolute ethanol (400 ml.) was added with stirring and the precipitate allowed to settle. The coagulated proteins were filtered off and washed with a small volume of 80 % (v/v) aqueous ethanol. The ethanolic extract was then allowed to stand for a few days at + 2°, when it was refiltered and evaporated to 60 ml. under reduced pressure. Part of the concentrated solution (20 ml.) was further evaporated under reduced pressure to 2 ml. (solution (*a*)). A second part (40 ml.) was passed through a column packed with activated 'Zeo-Karb 215' (wet volume 9 ml.) and the first 5 ml. of the effluent rejected. The effluent solution was then divided into two equal parts and one portion concentrated to 2 ml. (solution (*b*)). The remainder of the solution was passed through a column packed with prepared 'Deacidite' (wet volume 10 ml.), the first 6 ml. of the effluent being rejected and the remainder concentrated to 2 ml. (solution (*c*)). Passage through the zeo-karb column removed a yellow pigment from the solution and CO_2 was evolved; the effluent from the deacidite column was clear and colourless.

Chromatograms were prepared using phenol-NH_3 and butanol-acetic acid mixture as solvents, the three solutions being run side by side on the same paper strip. All three solutions showed well characterized spots corresponding to glucose (Pl. 3 (*c*)); fructose was not found. Grey-brown irregular spots due to interfering substances were visible with (*a*) and with (*b*) but were absent from (*c*).

Detection of fructose, glucose and inositol in the foetal blood of the sheep. The presence of fructose as well as glucose in the foetal blood of the sheep was demonstrated by Bacon & Bell (1946) who isolated the 2 : 3, 4 : 5 diacetone derivative of fructose (Bell, 1947). A laevorotatory sugar had previously been reported by Paton, Watson & Kerr (1907). An extract of whole foetal blood that had been deproteinized by ethanol-chloroform treatment was kindly supplied by Dr J. S. D. Bacon. This was prepared from 50 ml. of blood and contained, in addition, water (150 ml.), ethanol (100 ml.) and chloroform (50 ml.). The solvents were removed by evaporation and the solution made up to 40 ml. A portion of the aqueous solution (20 ml.) was passed through a column containing activated zeo-karb (3 g.) and then through deacidite (5 g.), following the procedure described above for white of egg. The salt-free effluent from the columns was concentrated under reduced pressure to a volume of 2–3 ml., after which it was evaporated to dryness under vacuum at room temperature and the dry residue made up to 0·25 ml. with distilled water.

Chromatograms in three solvents, phenol, collidine and butanol-acetic acid, showed the presence of glucose, fructose and inositol when sprayed with the ammoniacal silver nitrate reagent. Since the spot due to inositol was light in colour, further chromatograms were sprayed with ammoniacal silver nitrate containing NaOH to a concentration of 0·05 N, and in these the inositol showed strongly in the mixture and had R_F values identical with the values given by authentic specimens. Further chromatograms run in phenol and butanol-acetic acid were sprayed with naphthoresorcinol-trichloroacetic acid mixture. These showed the presence of fructose as red spots of R_F value identical with those due to authentic specimens of the sugar.

The procedure given above was repeated with samples of blood from three normal adult sheep (not pregnant), and in each case glucose only was found; fructose and inositol appeared to be absent or in quantities too small to be detected.

DISCUSSION

In Fig. 1 the R_F values of the reducing sugars in phenol-NH_3 have been plotted as ordinates and the values in *s*-collidine as abscissae. The circles drawn around each point give an approximate indication of the relative size of the coloured spot normally encountered, so that the figure gives a general indication of the degree of separation obtainable with the two solvents.

With two solvents only, complete separation between several pairs of sugars is not obtainable, but other solvents and solvent mixtures allow of further separation. A useful third solvent is *n*-butanol-acetic acid mixture, and experience has shown that it is usually necessary to run chromatograms with at least three solvents if unambiguous identifications are to be ensured.

With none of the solvents tested has complete separation been obtained on 18–20 hr. chromatograms between sorbose and glucose, arabinose and fructose or chondrosamine and glucosamine, so that clear identification is not yet possible when both sugars of any of these pairs appear in a solution together. However, when only one sugar of such a pair is present the separation is usually sufficient for successful identification by reference to the R_F values in several solvents.

Some inference as to the effect of chemical constitution on R_F values may be drawn from the spread of the sugar spots as displayed in Fig. 1. In general, as would be expected, the more strongly hydrophilic substances are the more slowly transported. The carboxylic acid group is particularly effective in giving rise to a low R_F value as indicated by the slow movement in both solvents of the uronic acids. When an —OH group is replaced by —NH_2 as in the hexosamines, a higher R_F value results with phenol but not with collidine. Still higher values with both solvents may be obtained by replacement of —OH by H as in the deoxysugars.

The aldohexoses occupy roughly the centre of the scale, but overlap with the ketoses. The pentoses appear to be rather faster and the disaccharides maltose and lactose are slower. However, it is characteristic of the results that the sugars most difficult to separate are not necessarily members of the same group in the usual chemical classification. The behaviour of dehydroascorbic acid appears to be anomalous, since it is slow in phenol, but has a very high mobility in collidine. This may be due to the intervention of the lactone structure.

The bearing of various external conditions upon the reproducibility of the R_F values of the amino-acids has been discussed by Consden *et al.* (1944), and since the same considerations apply with equal force to the behaviour of reducing sugars the subject need not be considered in detail here. It is sufficient to point out that variations in R_F value due to changes in temperature and other factors may be considerable, and in the analyses described in the experimental section identification of the components of a mixture was always based on the R_F values of authentic specimens of the sugars when run side by side on the same filter-paper strip. In order to compensate for temperature variations, a standard sugar (glucose) was put up on every chromatogram, and all R_F values were corrected to 20° by reference to the standard sugar.

It is apparent from inspection of Fig. 1 that where more than a small number of reducing sugars appear in the same solution there would be an advantage in carrying out two-dimensional chromatograms, following the technique described by Consden *et al.* (1944). A few such chromatograms have been prepared, the spot containing the mixed sugars being developed in one direction with phenol and afterwards in a direction at right angles with collidine. The neutral reducing sugars gave encouraging results, but it was found that in the case of amino sugars too much spreading of the spot took place, with the result that the sugars could not be identified accurately by reference to their R_F values. The two-dimensional technique appears to be less reliable than the filter-paper strip method where the test solution contains a small concentration of one component, and since more than two or three different sugars are rarely found in natural mixtures, it has generally been considered preferable to apply the filter-paper strip method rather than the more time-consuming two-dimensional technique.

The R_F values given by the sugars are little affected by the presence of other sugars even though they may appear in the test solution in much larger quantities; in one experiment, glucose showed its normal R_F value in the presence of a concentration of lactose 50 times greater. Interference effects by electrolytes however are serious, and in carrying out the analysis of solutions of biological origin containing sugars it is almost always necessary to effect the prior removal of salts, acids and bases. A convenient way in which this may be done is by making use of ion-exchange reagents. For this purpose two synthetic ion-exchange resins were found to be suitable. Cations may be removed from neutral solution by passage through a column of the Permutit product 'Zeo-Karb 215' (Permutit Co., London), while the anions may be removed from the resulting acidic solution by similar treatment with 'Deacidite'.

The neutral reducing sugars give rise to essentially circular spots with sharp edges under the conditions described, and although various tautomeric reactions are possible with these substances, such reactions do not appear to affect the transport of the sugar as a single component. This is not altogether the case with the amino sugars or the uronic acids. The hydrochlorides of glucosamine and chondrosamine form short trails with acidic or neutral solvents but give well formed spots under conditions of sufficient alkalinity to suppress the cation. The uronic acids show a tendency to form heart-shaped spots with basic solvents, and in the case of glucuronic acid behaviour is complicated by the presence of a slow reversible reaction between free glucuronic acid and its lactone. In acidic solvents solutions of glucuronic acid give rise to two well separated spots, one of them due to the lactone. Galacturonic acid, on the other hand, does not readily form a lactone, and for this reason it can be differentiated from glucuronic acid in spite of the fact that the R_F values of the free acids lie close together.

A number of compounds not strictly belonging to the sugar series were briefly investigated, since they may be met with in the analysis of extracts of biological origin. Three such compounds were ascorbic acid, dehydroascorbic acid and inositol; however, it was found that although all three substances travelled as fairly well defined spots, the R_F values were well outside the range occupied by the reducing sugars. Ascorbic acid showed a very characteristic reaction with the ammoniacal silver nitrate reagent, giving an immediate black coloration in the cold, while, on the other hand, inositol reduced the silver reagent very slowly at 110°, and could only be detected when present in rather high concentration. As examples of the application of the technique, a number of extracts of biological origin have been examined, and three such analyses are reported in detail in the experimental section. Glucose, fructose and sucrose were identified in an 80 % (v/v) ethanolic extract of Bramley's Seedling apples, while an examination of the soluble sugars of fresh white of egg showed the presence of glucose only, no fructose being detected. In an ethanol-chloroform extract of whole blood from the foetus of a sheep the presence of fructose and glucose was confirmed, and in addition inositol was found. In all three cases it was necessary to remove interfering electrolytes by treatment with ion-exchange reagents.

The second part of this paper (this vol. p. 251) describes two examples of the application of the method to the analysis of the hydrolysis products of polysaccharides.

SUMMARY

1. A method is described for the identification of reducing sugars in mixtures in which use is made of the filter-paper chromatogram introduced by Consden *et al.* (1944).

2. The use as a spraying reagent of a solution of silver nitrate in ammonia was found to be effective as a general method for revealing the position of the sugars on the chromatogram, and other spraying reagents useful in special circumstances are described.

3. The behaviour on the filter-paper chromatogram of twenty-two sugars and sugar derivatives has been investigated using several solvents, and the degree of separation obtained has been indicated.

4. The effect of interfering electrolytes is discussed, and a method of removing them by application of ion-exchange reagents is described.

5. As examples of the technique, the analysis of three extracts of biological origin is described; glucose only was found in an 80 % (v/v) ethanolic extract of fresh white of egg, while glucose, fructose and sucrose were shown in a fraction from Bramley's Seedling apples. The presence of glucose, fructose and inositol was shown in an extract from the foetal blood of the sheep.

The author wishes to express his thanks to Dr D. J. Bell and Dr J. S. D. Bacon for their interest in the work, and for the gift of some of the specimens of pure sugars used in this investigation.

This work forms part of the programme of the Food Investigation Board of the Department of Scientific and Industrial Research.

CORRECTION ADDED 12 MARCH 1948

The R_F values for dehydroascorbic acid given in Table 2 above were noted to be anomalous and reinvestigation has revealed an error due to the interference of I′ from NaI in the solution. Although NaI gives no brown colour when sprayed with $AgNO_3$-ammonia under the conditions used, it has now been shown that on irrigation with the chromatographic solvents the ions of the salt migrate at different speeds, each forming a discrete spot on the paper. On spraying with $AgNO_3$-ammonia the reagent reacts with HI derived from I′ to give an intense purple-brown colour. Cl′ is inactive, but Br′ gives a blue-grey colour when present in high concentration.

The R_F values for dehydroascorbic acid should be corrected as follows: collidine, 0·47; phenol (additions: NH_3 (1 %), HCN) 0·28; butanol-acetic acid, 0·48.

Addendum. Note on the Behaviour of Inorganic Salts on the Filter-paper Partition Chromatogram

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During the course of some work on the dialyzable fraction of fresh rabbit muscle, the chromatographic method described in this paper was applied to the identification of the reducing substances. Application of the routine procedures detailed above led to the expected identification of glucose, but in addition an unknown substance was present which gave rise to a heavy spot on the chromatogram after spraying with $AgNO_3$-ammonia. The substance had an R_F value of 0·59 in phenol-1 % NH_3, 0·09 in *s*-collidine and 0·20 in butanol-acetic acid mixture and was adsorbed by the cation exchange reagent 'Zeo-Karb 215'. It was therefore considered to be basic in character.

The behaviour on the filter-paper chromatogram of a number of bases known or suspected to be present in muscle juice was then investigated. The R_F values and the spraying reagents used to reveal the positions of the spots are given in Table 1. The reducing substances that were responsible for the reduction of the silver reagent.

Neutral inorganic salts, when introduced on to a test sheet of filter paper, gave no colour with $AgNO_3$-ammonia after similar heat treatment, but if applied to a chromatogram and irrigated with phenol-1 % NH_3 they gave rise to heavy black spots after removal of the solvent and spraying with the reagent. This effect appeared to be due to hydrolysis of the salt with separation of the resulting strong acid and strong base. It was found that the area containing free base could be demonstrated by application of a number of common indicator dyes, a solution of bromothymol blue in a mixture of butanol and ethanol being particularly useful as a spraying agent. Solutions (0·01 M) of NaCl, KCl, $MgCl_2$, $CaCl_2$ and $BaCl_2$ were applied as spots to two filter-paper strips, and both strips were irrigated with phenol-1 % NH_3 (18 hr.). After removal of the solvent at 105°, one strip was sprayed with bromothymol blue solution and the other with $AgNO_3$-ammonia. The paper sprayed with the indicator showed a series of dark blue spots on a green ground, while that sprayed with the silver reagent gave a corresponding series of brown spots. The spots had short backward trails, but the leading edges were sharp. There was little differentiation of R_F values, all the salts giving rise to alkaline areas at R_F 0·55–0·58. The amount of salt applied to the chromatogram was very small, being in each case not more than 2–4 μg.

Table 1. *R_F values of some substances present in muscle*

Substance	Phenol-NH_3	*s*-Collidine	Butanol-acetic acid	Reaction with $AgNO_3$-NH_3	Reagent used for spraying	Colour of spot
Anserine	0·92	0·10	0·18	0	Ninhydrin	Red-brown
Carnosine	0·83	0·09	0·20	0	Ninhydrin	Red-brown
Creatine	0·82	—	—	0	Picric acid reagent	Orange
Creatinine	0·94	—	—	0	Picric acid reagent	Orange
Urea	0·73	0·44	0·43	0	Ehrlich's reagent	Grey
Spermine	0·95	0·01	0·03	+	Bromothymol blue	Blue
Spermidine	0·95	0·03	0·07	+	Bromothymol blue	Blue
Glucose	0·39	0·39	0·18	+	$AgNO_3$-NH_3	Brown
NaCl-KCl	0·59	0·09	0·20	+	Bromothymol blue	Blue

strong bases spermine and spermidine gave brown spots when treated with $AgNO_3$-ammonia, but it was also found that strong inorganic bases, which would not be expected to reduce silver nitrate, gave similar brown colorations. In order to demonstrate this effect, various dilutions of NaOH and KOH were introduced in spots as duplicates on two strips of filter paper. One strip was heated at 105° for 1 hr. (the normal time allowed to remove the solvent from a filter-paper chromatogram), while the other was not heat treated. Both strips were sprayed with $AgNO_3$-ammonia and placed in the oven for 10 min. to develop the colour. The spots on the heat-treated paper reacted strongly to the silver reagent, whilst those on the control strip reacted only slightly. From this it appeared that the effect of the alkali was to modify the cellulose of the paper during the heat treatment required to remove the solvent from the chromatogram. This resulted in the liberation of

Not all indicator dyes were suitable for use in showing the presence of acids and bases on the chromatograms, since the behaviour of the dyes appeared to be affected by the cellulose itself, and by the presence on it of residual traces of the solvent used in developing the chromatogram. Suitable dyes were found by trial and error. Bromothymol blue

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was satisfactory for strong bases both with phenol-1% NH_3 and with collidine as chromatographic solvents; in the case of collidine chromatograms the alkaline spots showed blue on a yellow ground.

The identity of the unknown spot on the chromatograms of muscle juice and the spots given by chlorides of sodium and potassium was shown by a comparison of the chromatograms in three different solvents, phenol-1% NH_3, collidine and butanol-acetic acid mixture. When the solutions were developed side by side on the same paper strips, identical R_F values were obtained. The spot given by the inorganic salts in muscle juice was sufficiently intense to react strongly with the bromothymol blue indicator, and this reagent has since been used to confirm the basic character of other spots suspected as being due to interference by inorganic salts. However, for the purpose of identifying the reducing sugars in a mixture, it is more satisfactory to ensure the removal of all but traces of inorganic salts by preliminary treatment with suitable ion-exchange reagents.

The process of separation of the ions of a salt on the paper chromatogram may be regarded as analogous with that occurring on ion-exchange reagents, and is of particular interest since both products of hydrolysis of the salt appear as narrow moving bands.

A simple explanation of the phenomenon lies in a consideration of the equilibrium

$$NaCl + C_6H_5OH \rightleftharpoons C_6H_5ONa + HCl.$$

Here, undissociated sodium phenate would be expected to have a high R_F value in phenol, and the equilibrium would be moved to the right as a result of transport of sodium phenate out of the system. A mechanism of this sort is rendered plausible, since in phenol-NH_3 and butanol-acetic acid the sodium ion travels farther than the chloride ion, while in the basic solvent collidine the reverse is the case. Table 2 shows the separation of sodium and chloride ions obtained with a number of solvents, and it will be observed that in phenol-NH_3 the R_F values of Na^+ and Cl^- from sodium chloride are identical with the values given by Na^+ and Cl^- from sodium hydroxide and hydrochloric acid. In butanol-acetic acid and *s*-collidine, separation is not so complete, and the spots due to Na^+ and Cl^- from NaCl occupy intermediate positions between those due to the same ions from NaOH and HCl. The presence of ammonia vapour has a marked effect on the separation taking place with phenol as solvent, since in its absence separation is very poor, and with sodium chloride only light trails due to NaOH and HCl are detectable.

Table 2. *R_F values given by* Na^+ *and* Cl^- *with various solvents*

	Phenol-NH_3		*s*-Collidine		Butanol-acetic acid	
	(Na^+)	(Cl^-)	(Na^+)	(Cl^-)	(Na^+)	(Cl^-)
NaOH	0·61	—	0·07	—	0·23	—
NaCl	0·61	0·17	0·15	0·38	0·20	0·15
HCl	—	0·17	—	0·43	—	0·25

The possibility of the intervention of cellulose itself, acting as an ion-exchange reagent, should be taken into account in discussing the mechanism of the separation, and the distribution of the ions between the phases will also be affected by the potential existing at the surface of shear between the wet cellulose and the moving solvent. It is probable, therefore, that the explanation suggested above presents a considerable over simplification of the problem.

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Filter-paper Partition Chromatography of Sugars

2. AN EXAMINATION OF THE BLOOD GROUP A SPECIFIC SUBSTANCE FROM HOG GASTRIC MUCIN AND THE SPECIFIC POLYSACCHARIDE OF *BACTERIUM DYSENTERIAE* (SHIGA)

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The primary object of this investigation was to assess the value of the chromatographic technique described in the preceding paper (this vol. p. 238) as a qualitative method for the analysis of the hydrolysis products of complex polysaccharides. Interest especially centres in the general class of mucopolysaccharides—polysaccharides which contain hexosamines and which frequently show a special capacity for forming combinations with proteins—the reactions of which may be of considerable importance in the storage behaviour of foodstuffs. The specific polysacharide prepared from the 'O' somatic antigen of *Bacterium dysenteriae* (Shiga) and the blood group 'A substance' from hog gastric mucin were chosen as being polysaccharides of this class which were available in a condition of presumed homogeneity and which had already been investigated by the classical methods.

The occurrence of complex polysaccharides having blood group A activity in certain mucinous secretions of man and animals has been dealt with in recent reviews by Wiener (1943) and Morgan (1945). A chemical investigation of the glycoproteins of hog gastric mucin was undertaken by Meyer, Smyth & Palmer (1937), who found the neutral carbohydrate portion of their product to consist of equimolecular proportions of galactose and *N*-acetylglucosamine. Later, Landsteiner & Hart (1940) showed that the material had blood group A activity and contained a component rich in amino-acids.

Morgan & King (1943) and Morgan (1944) extended these observations by preparing a highly purified and essentially undegraded specific substance from hog gastric mucin which contained some 15 amino-acids in addition to the sugar residues.

The constitution of the carbohydrate portion of the complex was further studied by Bray, Henry & Stacey (1946 *a*, *b*) who isolated material showing blood group A activity from a number of different sources. The group A specific substance from pepsin was studied by the method of methylation and hydrolysis, with subsequent isolation of the methylated sugars. Four sugar constituents were found, viz. glucosamine, galactose, fucose and mannose. A parallel experiment was carried out with the blood group 'A substance' isolated by Morgan & King from hog gastric mucin, and from this substance also a fully methylated L-fucose derivative was obtained in 6% yield, but the remaining sugar constituents are not stated.

It has since been pointed out (Aminoff, Morgan & Watkins, 1946) that the apparently homogeneous specimens of 'A substance' isolated from commercial hog gastric mucin possess both A and O blood group specificity. Mucoids with A or O specificity only may be prepared from the stomachs of individual hogs and it is probable that commercial hog gastric mucin, which is derived from many animals, contains a mixture of two mucoids of very similar chemical and physical properties but of differing immunological specificity.

The sample of bacterial specific polysaccharide used in the present investigations was supplied by Dr W. T. J. Morgan. It was obtained from the somatic 'O' antigen of *Bact. dysenteriae* (Shiga) (Morgan, 1936, 1937) by gentle hydrolysis with acetic acid, and has been shown to contain L-rhamnose, D-galactose and an acetylhexosamine believed to be *N*-acetylglucosamine (Morgan, 1938).

EXPERIMENTAL

Blood group A substance

The sample of blood group A substance was kindly supplied by Dr W. T. J. Morgan. It had been obtained from commercial hog gastric mucin by extraction with phenol and was shown to be electrophoretically homogeneous at pH 4 and 8. On hydrolysis with 0·5 N-HCl at 100° the reducing sugar reached its maximum after 4 hr., when the reducing value (expressed as glucose) was 50%.

The substance (30 mg.) was sealed with 1·5 ml. of 0·5 N-H_2SO_4 in an ampoule and heated for 4 hr. at 100°. 1 ml. of the hydrolysate was brought to pH 4·5 with $Ba(OH)_2$ solution and the $BaSO_4$ removed by centrifugation. The clear solution, together with the washings, was concentrated to dryness under vacuum at room temperature and made up to 0·5 ml. in readiness for application to the chromatograms. Phenol-1% (w/v) NH_3, *s*-collidine and butanol-acetic acid mixture were used as solvents for the chromatograms and glucose, D-glucosamine hydrochloride, chondrosamine hydrochloride, L-fucose, D-galactose and D-mannose were included as reference sugars.

s-Collidine. The chromatograms gave four spots corresponding to fucose, galactose, glucosamine and an unknown reducing substance giving a well defined circular spot at R_F 0·26. Mannose was absent.

Phenol-1% (*w*/*v*) NH_3. The chromatogram showed three well defined spots corresponding to fucose, glucosamine and galactose. Chondrosamine was absent. Mannose was not detectable since it would be obscured by galactose.

Butanol-acetic acid. The chromatogram gave clear indication of fucose, galactose and glucosamine. Mannose was absent, but an unknown reducing substance yielded a spot at $R_F = 0·055$.

The principal sugars present were galactose and glucosamine, fucose appeared to be present in smaller amount. In case hydrolysis had not been completed under the conditions used for the above series, a further sample of the polysaccharide was hydrolyzed for 10 hr. with $N\text{-}H_2SO_4$, and the hydrolysate treated as before. The resulting chromatograms were similar to those previously obtained except that the spot given by fucose was relatively lighter, and that given by glucosamine was heavier.

The R_F value of the unknown reducing substance was below the range normally encountered for hexoses, pentoses and amino sugars, and the presence of a reducing disaccharide was considered unlikely since the unknown spot persisted after hydrolysis under more vigorous conditions.

Treatment with cation-exchange resin. The hydrolysate from 30 mg. of the polysaccharide (contained in 3 ml. solution) was shaken for 30 min. with an activated and washed sample of 'Zeo Karb 215' (0·5 g.) as described in the previous communication. The concentrated filtrate was then applied to further chromatograms. The treatment with ion-exchange resin resulted in removal of the unknown material together with glucosamine, and the chromatograms in all three solvents showed two spots only, corresponding to galactose and fucose (Pl. 4 *a*). It is probable, therefore, that the unknown substance is basic in character.

The experiments described above indicate clearly the presence of galactose, glucosamine and fucose, but give no indication of the presence of mannose in the sample of blood group A substance used for the investigation.

The specific polysaccharide of Bact. dysenteriae (*Shiga*)

The polysaccharide had N 1·6% and yielded 97% of reducing substances calculated as glucose after acid hydrolysis (Hagedorn & Jensen). A solution of the material (10 mg.) in $0·5N\text{-}H_2SO_4$ (2 ml.) was sealed in an ampoule and heated at 100° for 5 hr. The hydrolysis mixture was clear and colourless and a measured volume (1 ml.) was neutralized to pH 4·5 with $Ba(OH)_2$ solution. After removal of the $BaSO_4$ the solution, together with the washings, was transferred to a small crucible and evaporated to dryness under vacuum at room temperature. The dry residue was dissolved in water (0·1 ml.) and the solution stored at +2° over chloroform. Where one-dimensional chromatograms were applied, the following sugars were included as reference substances; glucose, D-glucosamine hydrochloride, *N*-acetylglucosamine, chrondrosamine hydrochloride, L-rhamnose and D-galactose. Collidine, phenol-1% (w/v) NH_3 and butanol-acetic acid mixture were used as solvents.

s-Collidine. The developed chromatogram showed two spots. One of them at R_F 0·59 corresponded to rhamnose, while the second spot at R_F 0·32–0·34 appeared to be multiple. The slower moving substance gave rise to a black coloration with $AgNO_3$-ammonia as soon as the temperature of the oven reached 90° and later (at 105°) the spot extended in size with development of a brown colour.

It was considered that the multiple spot at R_F 0·32–0·34 was probably due to both glucosamine and galactose, and for this reason the experiment was repeated using a spraying reagent, based on the Morgan & Elson colour reaction for hexosamines. (Details are given on p. 240 of this volume.) In order to show the presence of *N*-acetylhexosamines, a chromatogram was sprayed with the modified Ehrlich's reagent alone, while to show both free hexosamine and the *N*-acetylhexosamines a second chromatogram was sprayed with the alkaline acetylacetone reagent, followed by the acid Ehrlich's reagent. Under these conditions the spot at R_F 0·33 showed the presence of free glucosamine in small quantity while a third spot appeared at R_F 0·50 corresponding to *N*-acetylglucosamine.

Phenol-1% (*w*/*v*) NH_3. With this solvent galactose was shown unambiguously, but the rhamnose spot was confused with that due to glucosamine (Pl. 4 *b*). As before both free glucosamine and *N*-acetylglucosamine could be detected as red-violet spots by means of the modified Morgan & Elson reagents.

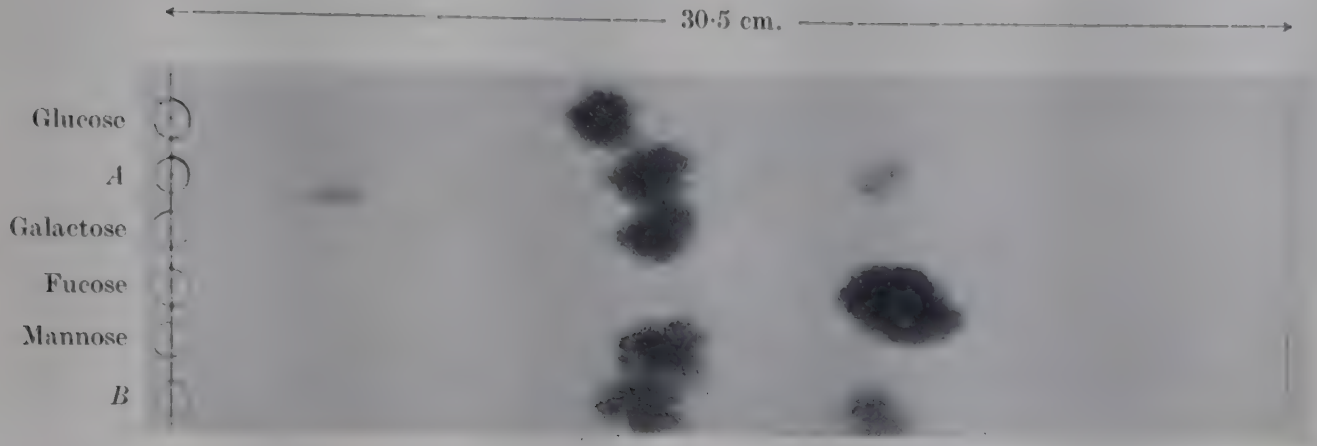
Butanol-acetic acid. Like collidine this solvent mixture led to the unambiguous identification of rhamnose, but as in the case of collidine, the spot due to glucosamine was confused with that due to galactose.

Since *N*-acetylglucosamine was found it was evident that the conditions of hydrolysis were not sufficiently severe to complete the deacetylation of the amino sugar. For this reason the experiments described above were repeated, hydrolysis being carried out with $N\text{-}H_2SO_4$ for 10 hr. at 100°. The hydrolysate no longer showed the presence of *N*-acetylglucosamine, and the spots due to free glucosamine were correspondingly heavier. Galactose and rhamnose appeared in much the same concentration as before.

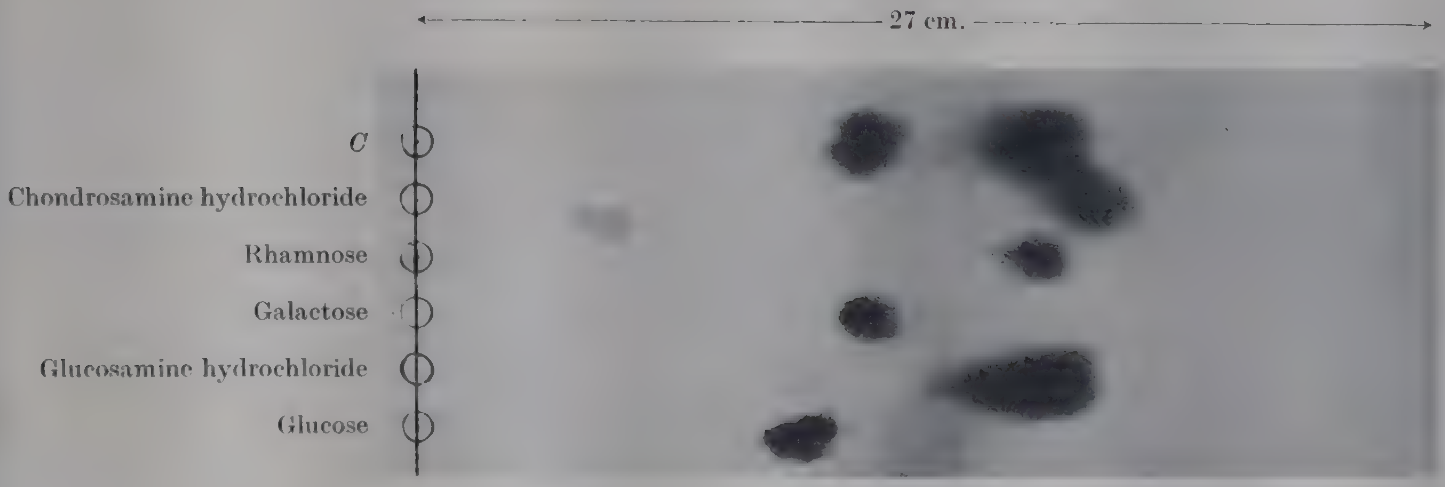
As confirmatory evidence, a two dimensional chromatogram was set up with a hydrolysis mixture prepared by heating the polysaccharide with $N\text{-}H_2SO_4$. The chromatogram was run first for 24 hr. with phenol-1% (w/v) NH_3, and after drying turned through 90° and irrigated with collidine for a further period of 24 hr. The chromatogram showed two heavy spots, which could be identified from their co-ordinates as galactose and rhamnose. The glucosamine appeared as a diffuse trail which was unsuitable for the purpose of identification from co-ordinates.

DISCUSSION

As would be expected in the case of a homogeneous polysaccharide, the identification of the sugars present in the product of hydrolysis of the specific substance from *Bact. dysenteriae* (Shiga) was uncomplicated by the presence of interfering substances; galactose, glucosamine and rhamnose could readily be recognized by reference to the respective R_F values in each of three solvents. The presence of the hexosamine was confirmed by spraying the developed chromatograms with alkaline acetyl-


30·5 cm.
Glucose
A
Galactose
Fucose
Mannose
B

(*a*) Hydrolysate of blood group A specific substance after treatment with ion-exchange resins; showing the presence of galactose and fucose. The hydrolysate after treatment with 'Zeo-Karb 215' was placed in the circle marked '*A*'. *B* shows position of a hydrolysate treated with both 'Zeo-Karb' and 'Deacidite'. Chromatogram run with phenol for 20 hr.


27 cm.
C
Chondrosamine hydrochloride
Rhamnose
Galactose
Glucosamine hydrochloride
Glucose

(*b*) Hydrolysate of polysaccharide from *Bact. dysenteriae* (Shiga): showing presence of galactose and a multiple spot due to rhamnose and glucosamine. The hydrolysate was placed in position marked '*C*'. Chromatogram run with phenol for 16 hr.

acetone followed by an acid solution containing *p*-dimethylaminobenzaldehyde, and as further confirmation the presence of galactose and rhamnose was shown by carrying out a two-dimensional chromatogram according to the technique described for amino-acids by Consden, Gordon & Martin (1944).

With the blood group A substance, the analysis was complicated by the fact that only some 70% of the material was carbohydrate in nature, and an interfering substance was found which gave rise to a spot at R_F 0·26 in collidine after spraying with the ammoniacal silver nitrate reagent. This substance was removed by treatment with a cation-exchange resin, and was therefore considered to be basic in nature. The analysis showed clearly the presence of glucosamine, galactose and fucose, but, in spite of indications in the literature of the presence of mannose in allied blood group A substances, this sugar was not found in the hydrolysis products of the polysaccharide under investigation.

The analysis of these two trial substances indicates clearly the importance of control of the hydrolysis conditions, and in assessing the results of such analyses it should be borne in mind that the hydrolysis of complex polysaccharides may proceed in stages, some destruction of the less stable sugar residues taking place before complete liberation of residues constituting a resistant core.

SUMMARY

1. The method of separating reducing sugars on filter-paper partition chromatograms described in this volume p. 238 has been applied to the analysis of the products of hydrolysis of two carbohydrate-containing materials: (*a*) the specific polysaccharide of *Bact. dysenteriae* (Shiga), and (*b*) the blood group A substance from hog gastric mucin.

2. The results of the analysis of (*a*) agree with results in the literature in showing the presence of glucosamine, galactose and rhamnose.

3. In the hydrolysis products of (*b*) glucosamine, galactose and fucose were found. Mannose, which has been observed in active polysaccharide fractions from other sources, was not found in the specific substance from hog gastric mucin.

The author wishes to express his thanks to Dr W. T. J. Morgan for his interest and for the gift of the polysaccharides used in this investigation.

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Urinary Elimination of Synthetic Oestrogens and Stilboestrol Glucuronide in Animals

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Clinical experience has shown that oestrogen treatment of carcinoma of the prostate fails after a varying lapse of time in many cases. Although a similar delayed recurrence of symptoms also frequently follows treatment of prostatic carcinoma by castration (Angrist & Khoury, 1944; Nesbit, Pazzos & Cummings, 1944; Huggins, 1946) there is a slight possibility that the failure of continued oestrogen treatment (in spite of increased dosage) is due to some bodily adaptation involving an altered metabolism of the drug. Hence it was thought worth while to study the metabolism of synthetic oestrogens in patients and animals; at the same time the opportunity has been taken to collect the monoglucuronides of dienoestrol (4:4′-dihydroxy-γ:δ-diphenylhexa-β:δ-diene), hexoestrol (4:4′-dihydroxy-

γ:δ-diphenyl-n-hexane), and stilboestrol (4:4′-dihydroxy-α:β-diethylstilbene) for chemical and biological investigation (Simpson & Wilder Smith, 1948).

The metabolism of the natural oestrogens has been widely studied, but that of the synthetic oestrogens comparatively little. Stroud (1939) found that in the rabbit 21·3 % of a high dose of hexoestrol was excreted in the urine, 15·7 % in the free state and 5·6 % in a conjugated form. The corresponding figures for stilboestrol were 14·2 and 4·2 %, and for dienoestrol 11·0 and 3·0 %. In view of the instability of dienoestrol (Wilder Smith & Williams, 1945; 1947) this low figure is not surprising as the vigorous conditions probably caused some decomposition. No attempt was made to isolate the conjugated oestrogen as such, and in no case was more than 25 % of the total administered oestrogen accounted for.

Mazur & Schorr (1942) dosed rabbits with stilboestrol and isolated from the urine 30 % of the administered oestrogen as the monoglucuronide. No other synthetic oestrogens were investigated and no other metabolic products were reported.

We have found that in the rabbit a high percentage of the synthetic oestrogen given is excreted as glucuronide or other derivative soluble in sodium bicarbonate, while quite a small amount is excreted as free oestrogen. Much lower total excretion was found in the cat.

METHODS

Urine collection and extraction. The urines were treated as described by Simpson & Wilder Smith (1948), care being taken to avoid hydrolysis of glucuronide by heat or by bacterial growth. After acidification of the urine to Congo red with dilute HCl in the cold, ether extraction (by hand and continuous) yielded an extract which gave a fraction, soluble in bicarbonate, containing glucuronide, which was characterized as described by Simpson & Wilder Smith (1948), and a fraction soluble in caustic soda, containing free oestrogen. The latter extract was made acid to Congo red, extracted with ether and the extract assayed biologically, giving the yield of free oestrogen excreted. As an additional check, a sample of the original unextracted urine was tested for oestrogenic activity: this gave an indication of the combined activity due to free and conjugated oestrogen. After the sodium oestrogen glucuronidate had crystallized from the bicarbonate solution (where this was used) the mother liquor was acidified, extracted with ether, and the ether extract assayed biologically; this eliminated errors due to incomplete crystallization. That the ether extraction of the acidified but unhydrolyzed urine was complete was proved in several experiments by hydrolyzing afterwards, and extracting the urine with ether in a continuous extractor. No oestrogens could be detected either in the bicarbonate or caustic soda fractions from the ether extract. Thus complete extraction of both free and conjugated oestrogens had been attained (see below, p. 256).

Biological assay and expression of results. Usually the amount of oestrogen present in the urine was so great that the crude urine could be assayed without any preliminary extraction. In other cases the various fractions of the urine during extraction were assayed to serve as a check on the completeness of the chemical separations.

The method of assay was the one usual in this Institute: Spayed adult rats were injected twice daily for 3 days with the extract or urine to be tested and the vaginae of the rats were smeared twice daily during two following days. Smears consisting of epithelial and/or cornified cells only were taken to represent positive responses. The number of samples to be tested and the uncertainty of their activity made it impossible for more than 5–10 rats to be used at each dose level and the doses used were in the proportions 1, 2, 5, 10, 20, 50, 100, etc., so the results have only a very low degree of accuracy.

The content of the samples assayed biologically was estimated (on the assumption that all the activity was due to free oestrogen or to oestrogen glucuronide) by reference to dose-response curves for the substances concerned (Simpson & Wilder Smith, 1948). In either case and also where chemical separations were effected the content of free oestrogen was calculated and is expressed throughout as a percentage of the total dose given.

RESULTS

Elimination after subcutaneous injection into rabbits

The results summarized in Table 1 are derived from an experiment where four female rabbits were injected daily (except Sundays) with 30 mg. of dienoestrol or stilboestrol for 4 weeks.

The total proportion of the administered oestrogen eliminated is somewhat higher for stilboestrol than for dienoestrol. In this particular experiment the difference is not statistically significant but Stroud (1939) found the same difference, and in other experiments we have recovered as much as 46 % of administered stilboestrol as the glucuronide.

During the injection period the total elimination remained fairly constant. There was a slight tendency for the proportion of free oestrogen to decline after the first week (though the third week in rabbit 3/46 is exceptional) and with stilboestrol there may be a slight delay before the glucuronide elimination process becomes fully effective. It may be noted that the crystallization of the glucuronide from the sodium bicarbonate was rather more complete with dienoestrol than with stilboestrol (cf. columns 4 and 5, Table 1).

One of the most striking facts revealed is that the elimination of these high doses is so rapid. Although the injections were made in oily solution and oil remained unabsorbed at the end of the sixth week of the experiment, yet even in the week after the injections had stopped the excretion had dropped to low levels.

Table 1. *Urinary elimination of subcutaneously injected oestrogens*

(Six injections of 30 mg. of oestrogen each week for 4 weeks.)

		Percentage of total weekly dose eliminated			
			(ii) As glucuronide		
Rabbit	Week	(i) Free	Gravimetric	Biological*	Total
		Dienoestrol injections			
No. 1/46, ♀	1	5	16	1	22
	2	1	—	—	—
	3	1	14	5	20
	4	1	15	5	21
	5	0·1	3	<1	3
	6	—	—	<1	<1
No. 2/46, ♀	1	5	21	1	27
	2	1	18	4	23
	3	1	17	4	22
	4	1	22	3	26
	5	0·1	—	<1	<1
	6	—	—	<1	<1
		Stilboestrol injections			
No. 3/46, ♀	1	10	1	10	21
	2	5	19	10	34
	3	15	7	10	32
	4	1	10	10	21
	5	0·1	—	>1	>1
	6	—	—	<1	<1
No. 4/46, ♀	1	10	2	10	22
	2	10	—	10	20
	3	5	10	10	25
	4	5	13	10	28
	5	0·1	—	4	4
	6	—	—	<1	<1

* Biological assay of mother liquids after removal of gravimetrically estimated compound.

Elimination after subcutaneous injection into cats

Two female cats each received 1·8 g. of dienoestrol (dissolved in sesame oil with a little added ethanol) by subcutaneous injection during 1 week. Their urine was collected during the week of the injections and the following week.

About 1 % of the injected oestrogen was excreted in the free form, but no glucuronide could be isolated. However, the fraction soluble in bicarbonate showed oestrogenic activity on biological assay, and if this activity were all due to glucuronide it would account for 4 % of the administered dienoestrol.

During the injection period the daily urine volume in the cats was abnormally low. A similar decrease had been noticed in the injected rabbits: in their case the decrease was due to the smaller consumption of cabbage, which was the chief component of their diet. It is well known that oestrogens in high doses depress the appetite and, although the fluid intake of the cats was not measured, we have little doubt that it was this which was responsible for the small output in the cats also.

The toxic effects of dienoestrol were much more marked in the cats than in the rabbits. Both the cats in this experiment died; one 2 weeks, and the other 3 weeks, after the first injection.

Effect of urinary pH *on elimination of injected dienoestrol by female rabbits*

A possible reason for the different rates of elimination of oestrogen glucuronide in cats and rabbits might lie in the difference in the reaction of their urine. That in the normally fed rabbit is alkaline while cats' urine is strongly acid.

To investigate this possibility two mature female rabbits were fed on a diet of oatmeal, bread and water until their urine was consistently acid to litmus (pH 5) and then injected subcutaneously with 1·5 g. of dienoestrol (dissolved in sesame oil) as before.

The results given in Table 2 show clearly that an acid urine does not prevent the elimination of large proportions of injected dienoestrol in the form of the glucuronide. Again, as with the experiments in rabbits with alkaline urine, the variations in glucuronide yield are wide.

Table 2. *Elimination of dienoestrol glucuronide in rabbits with acid urine* (pH 5) *after subcutaneous injection of dienoestrol*

	Dosage		Urine collection	
Rabbit	Time (weeks)	Amount (mg.)	Time (weeks)	Percentage of dienoestrol excreted as glucuronide
R 24/46	1–3	1500	1–7	24
R 25/46	1–3	1500	1–7	48

Table 3. *Elimination of hexoestrol glucuronide in rabbits after oral administration of hexoestrol*

		Days of		
Rabbit	Total dose (mg.)	(i) Dosing	(ii) Collection	Percentage of hexoestrol excreted as glucuronide
R 27/46	1000	1	1–9	30
R 31/46	700	1	1–4	46

Elimination after oral administration in rabbits

Single doses of hexoestrol, dissolved in aqueous ethanol or in sesame oil, were given by mouth to two female rabbits and their urine was collected (see Table 3).

The urine from R 27/46 was also assayed biologically. The activity found was equivalent to 5 % of the administered dose if it were all free hexoestrol, or to 90 % of the administered dose if it were all glucuronide. As this last figure is so much greater than the glucuronide isolated chemically it appears that some of the hexoestrol was, in fact, eliminated in the free form.

Similar experiments where two doses of stilboestrol, dienoestrol or hexoestrol were given 6 days apart gave similar results (Table 4).

Table 4. *Elimination of oestrogen after oral administration during 6 days of stilboestrol* (*S*), *hexoestrol* (*H*) *or dienoestrol* (*D*)

			Percentage of total dose eliminated			
				(ii) As glucuronide		
Rabbit	Total dosage (mg.)	Collection period (days)	(i) Free Biological	Gravimetric	Biological*	Total
R 32/47	1800 S	14	5	9	23	32
	Control†	14	<1	0	<1	<1
R 33/47	2000 H	11	<1	14	26	40
	Control	11	<1	0	<1	<1
R 30/47	1450 D	14	5	7	25	32
	Control	14	<1	0	<1	<1

* As in Table 1.

† Control collections were made after a lapse of 10 days from the end of the experimental collection.

In this experiment all the urine samples after ether extraction were hydrolyzed with hydrochloric acid under reflux and re-extracted with ether. These ether extracts were then worked up in the usual manner and biologically assayed. No oestrogenic activity was detectable in any of the extracts at the highest dose level tested (equivalent to a 1 % recovery of the oestrogen given). This proves that all the conjugated and free oestrogen had been extracted from the unhydrolyzed urine in the first place. The results again show that the oestrogen eliminated in the urine is predominantly conjugated.

Urinary elimination of stilboestrol glucuronide fed to the rabbit

Stilboestrol glucuronide (250 mg.) dissolved in sesame oil was fed by stomach tube to a female rabbit, the urine being collected and worked up as in the previous experiments. The striking feature of the results (Table 5) is that very low yields of glucuronide were obtained, showing that the glucuronide is certainly not the end product of metabolism. A second experiment in which 100 mg. of stilboestrol glucuronide dissolved in sesame oil were injected subcutaneously yielded similar results (Table 5).

The results of this experiment demonstrate that the glucuronide formed after the administration of stilboestrol is not a stable end product but is further metabolized in the body.

Table 5. *Urinary elimination of oestrogen after single dose of stilboestrol glucuronide*

Rabbit	Dose (mg.)	Collection period (days)	Percentage of total dose eliminated: (i) Free Biological	(ii) As glucuronide: Gravimetric	Biological*	Total
R 33/47†	250	4	1	12	1	13
R 7/47‡	100	4	3	0	25	25

* As in Table 1. † Oral administration. ‡ Subcutaneous injection.

DISCUSSION

Our results confirm those of Mazur & Schorr (1942) in demonstrating that rabbits excrete a high proportion of administered stilboestrol in the urine as glucuronide, but do not agree with the finding of Stroud (1939) that high proportions are excreted in the free form. This discrepancy is probably due to the method of working up used by Stroud. We have also found that dienoestrol and hexoestrol behave in the same way as stilboestrol, all yielding about 35 % of the administered dose as monoglucuronide in the urine. We have assumed that all the conjugated oestrogen is monoglucuronide as, in those cases where only 1 % remained for biological assay, the 99 % was in fact glucuronide. These conclusions relate to the very large doses we have used and are not necessarily applicable to lower, therapeutic doses.

The yields obtained in our earlier experiments were somewhat lower than those realized later; this is probably due to increased experience in handling these rather intractable substances. The main difficulty lies in the isolation of the glucuronides, either free or as sodium salts. This may be seen for example by the variable proportions of glucuronide remaining for biological assay in Tables 1, 4 and 5. Perhaps this can be attributed to the accumulation of various bicarbonate-soluble substances in the urine. If so, the time during which the urine is collected after dosage should be kept as short as possible, consistent with the recovery of the greater proportion of administered oestrogen; indeed the rapidity with which the dose is eliminated (see Table 1) renders any prolonged collection after dosage unnecessary.

The only stage so far identified in the metabolism of the synthetic oestrogens is that of glucuronide formation. We were unable to isolate any sulphate esters of the administered oestrogens and therefore have no direct evidence of their production in the rabbit. In man this form of elimination of natural oestrogens is well known and there is some indirect evidence of a small excretion of synthetic oestrogen sulphates (unpublished data).

It is very doubtful whether such metabolism studies can shed any light on the mechanism of oestrogenic action. Glucuronide and sulphate formation are too widespread to be specific for such action, while the complex degradation products of the natural oestrogens cannot be vital for oestrogenic action since the synthetic oestrogens produce the same effect without undergoing the same chemical changes. For closer study of oestrogenic action it would probably be advantageous to use the most chemically stable oestrogens available, e.g. hexoestrol (Wilder Smith & Williams, 1945, 1947) in order to avoid the complications of side reactions which are irrelevant to oestrogenicity.

SUMMARY

1. The urinary excretion of oestrogen after the administration of stilboestrol, hexoestrol, dienoestrol, and stilboestrol glucuronide has been examined in the rabbit and cat.

2. In the rabbit about 35 % of the administered oestrogen can be accounted for in the urine. In the cat much lower recoveries are obtained.

3. In the rabbit the chief metabolic products excreted are the monoglucuronides, but these substances are metabolized further to inactive products.

We are indebted to the Council of the Middlesex Hospital for the provision of laboratory facilities for this work and to Mrs Culliford and to Mr B. D. Shepherd for technical assistance. The investigation was aided by a grant from the Medical Research Council and by an apparatus grant from the Central Research Fund, University of London.

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1948

The Isolation and Properties of the Monoglucuronides of Stilboestrol, Hexoestrol and Dienoestrol

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The monoglucuronides of stilboestrol, hexoestrol and dienoestrol were isolated from the urine of rabbits during metabolism experiments described previously (Wilder Smith & Williams, 1948). Mazur & Schorr (1942) described the glucuronide of stilboestrol which they obtained from rabbits treated with stilboestrol. As far as we are aware the glucuronides of hexoestrol and dienoestrol have not been isolated before.

METHODS

Urine collection and extraction. The urine was collected twice daily in the presence of toluene and stored at 0–3°. In the dienoestrol experiments a few mg. of hydroquinone were added daily to the collection vessels to assist stabilization.

After acidification to Congo red with dilute HCl the urine was extracted by hand three times with an equal volume of ether. Within limits, the larger the volume of ether used for each extraction, the less danger there was of the formation of intractable foams. The remaining urine was then extracted in a continuous ether extractor for 24 hr. and the combined extracts dried over anhydrous Na_2SO_4. After concentration to 20–60 ml. the ether solution was set aside in the refrigerator to crystallize.

In the method used by Mazur & Schorr (1942) the ether extract was not dried, but was extracted with saturated $NaHCO_3$ solution from which the sodium salt of the glucuronide crystallized. This method gave somewhat low yields with stilboestrol and dienoestrol and in the case of hexoestrol yielded no glucuronide at all (except on one occasion). The free glucuronide sometimes crystallized well directly from the ether extract when in sufficiently high concentration, but where difficulty was encountered with dienoestrol or stilboestrol the $NaHCO_3$ method was used. In cases where this expedient yielded no crystalline sodium salt, the bicarbonate solution was acidified to Congo red, extracted with ether, the ether removed and the residue taken up in a small amount of acetone. Addition of the acetone solution to saturated aqueous $NaHCO_3$ usually yielded the crystalline sodium salt. Solution of the crude sodium salts in water followed by acidification in the cold with dilute HCl precipitated the free glucuronides as a plastic mass. After washing with a small volume of water, and drying *in vacuo* over $CaCl_2$, the crude solid was dissolved in a little acetone and filtered. On adding excess dry ether to the filtrate a slight amorphous precipitate was formed which was filtered off. On keeping the filtrate at 0–3° the glucuronide slowly crystallized.

It was not found possible to remove a molecule of water of crystallization from the hexoestrol glucuronide even after drying for 6 hr. *in vacuo* at 110°. Dienoestrol and stilboestrol glucuronides also hold water of crystallization tenaciously, which is, however, almost completely removed under the above conditions.

Biological assay. The oestrogenic activity of the glucuronides was tested by subcutaneous and intravaginal administration to spayed rats. The technique used for the assay by subcutaneous injection has been described previously (Wilder Smith & Williams, 1948). The intravaginal assay was carried out by the application of two doses of 0·01 ml. of oily solution into the rat vagina 24 hr. apart; smears were taken during the two succeeding days. In both cases positive responses were indicated by vaginal smears consisting of cornified and/or epithelial cells only and dose response curves were constructed and the ED_{50} calculated by the method of Fisher & Yates (1943).

RESULTS

Chemical properties

Stilboestrol monoglucuronide. Mazur & Schorr (1942) report the melting point, analysis and optical rotation of this substance. We have found very similar properties, which are given here for confirmation. In addition the physiological properties are reported.

After recrystallization of the white needles several times from dry ether containing a trace of acetone and drying 2 hr. *in vacuo* at 110°, white, semi-fused needles were obtained, softening 100–110°, melting with decomposition 174–176°. After longer drying periods some samples yielded a pale yellow syrup. On drying *in vacuo* (0·1 mm. Hg) at 110° for 4 hr. 10·7 mg. glucuronide lost 0·7 mg. (6·5 %) with extensive sintering. Theoretical loss for $1H_2O$, 3·9; for $2H_2O$, 7·5 %. On exposing the dried sample to the atmosphere for 48 hr. at room temperature, the glucuronide regained 0·3 mg. in weight. $[\alpha]_D^{24^\circ} = -51{\cdot}6^\circ$ ($c = 1{\cdot}60$ in absolute ethanol); $[\alpha]_D^{24^\circ} = -57{\cdot}6^\circ$ ($c = 0{\cdot}179$ in absolute ethanol). Equivalent weight = 451 (using 0·01 N-NaOH and methyl red as indicator). Theory = 444. Found: C, 64·1; H, 6·7. Calc. for $C_{24}H_{28}O_8$: C, 64·8; H, 6·4 %.

Hexoestrol monoglucuronide. This was obtained in the crude state by direct crystallization from an ether extract of the acidified urine. Difficulty was experienced in the purification of this substance since it did not crystallize as the sodium salt from saturated aqueous sodium bicarbonate solution, except in one experiment where the concentration of glucuronide used was higher than usual. Further crystallization either from acetone and benzene or from acetone and ether mixtures was sometimes difficult. It was therefore purified by dissolving in sodium bicarbonate solution, extracting with ether, making the aqueous layer acid to Congo red paper with dilute hydrochloric acid, extracting with ether and drying over anhydrous sodium sulphate. The ether was removed by distillation, and the residue taken up in a small amount of acetone and reprecipitated by addition of ether. The slight amorphous precipitate first formed was removed by filtration, when the monoglucuronide crystallized from the filtrate slowly on standing at 0°. The crystals thus obtained gave a positive Tollens test (after 30 min. acid hydrolysis).

After drying the fine white crystals for 4 hr. at 110° *in vacuo* a white mass of partially fused needles sintering at 85–95° and decomposing at 188–196° was obtained. On drying *in vacuo* (0·1 mm. Hg) at 110° for 4 hr. 7·9 mg. glucuronide lost 0·8 mg. (10·1 %) with some sintering. Theoretical loss for $3H_2O$, 10·8 %. On exposing the dried sample to the atmosphere for 48 hr. at room temperature the glucuronide regained 0·4 mg. in weight. The crystals were soluble in acetone and ethanol, sparingly soluble in ether, insoluble in light petroleum and benzene. $[\alpha]_D^{24^\circ} = -73{\cdot}4^\circ$ ($c = 0{\cdot}976$ in absolute ethanol). Equivalent weight = 482. Theory for the monohydrate of the monoglucuronide = 464. Found: C, 62·4; H, 6·7. $C_{24}H_{32}O_9$ requires C, 62·2; H, 6·9 %.

Dienoestrol monoglucuronide. This substance also proved difficult to purify since it did not crystallize well and was apparently somewhat unstable. The sodium salt of the glucuronide however crystallized from saturated aqueous bicarbonate solution and was used for purification. The free acid was soluble in acetone and ethanol, sparingly soluble in ether and insoluble in benzene and light petroleum and gave a positive Tollens test (after 30 min. acid hydrolysis). After drying 2 hr. *in vacuo* at 110° fine white needles were obtained, sintering at 105–115° and melting at 175–183° (with decomp.). On drying *in vacuo* (0·1 mm. Hg) at 110° for 4 hr. 9·9 mg. glucuronide lost 0·1 mg. (1·0 %) with little sintering. On exposing the dried sample to the atmosphere for 48 hr. at room temperature 0·1 mg. was regained in weight. $[\alpha]_D^{24^\circ} = -41{\cdot}5^\circ$ ($c = 0{\cdot}560$ in absolute ethanol). $[\alpha]_D^{24^\circ} = -47{\cdot}2^\circ$ ($c = 0{\cdot}182$ in absolute ethanol). Equivalent weight = 426. Theory for monoglucuronide = 442. Found: C, 64·4; H, 7·0. $C_{24}H_{26}O_8$ requires C, 64·8; H, 5·9 %.

Table 1. *Oestrogenic activity of stilboestrol, hexoestrol and dienoestrol monoglucuronides in rats*

(Figures for free oestrogens given for comparison in brackets.)

	ED_{50} (in μg.) with 95 % fiducial limits	
Oestrogen	By subcutaneous injection in aqueous solution	By intravaginal application in oily solution
Stilboestrol monoglucuronide	5·8 (5·5–6·2)	0·04 (0·03–0·05)
[Stilboestrol	0·35	0·003]
Hexoestrol monoglucuronide	11·5 (8·7–15·3)	0·07 (0·04–0·10)
[Hexoestrol	0·35	0·003]
Dienoestrol monoglucuronide	7·2 (6·5–8·1)	0·15 (0·07–0·3)
[Dienoestrol	0·75	0·003]

Biological properties

The oestrogenic activities of the three glucuronides are recorded in Table 1. The activities of the corresponding free oestrogens are given for comparison. Although aqueous glycerol is the preferred solvent for intravaginal tests, arachis oil was used in the reported tests for the sake of uniformity.

DISCUSSION

All the glucuronides have only 5–10 % of the oestrogenic activity of the corresponding free oestrogen. This is true whether the assays are made by subcutaneous or intravaginal methods.

If the activity were due to the complete hydrolysis and liberation of free oestrogen then the glucuronides should show about 50 % of the activity of their parent compounds. In actual fact their activity is about 10 %, calculated on the weight of oestrogen present. The small activity which the glucuronides do show may be due either to their intrinsically low oestrogenic potency, to the liberation of only a proportion of free oestrogen or to more rapid elimination from the organism. That the proportion of free oestrogen liberated, if any, is very small indeed is indicated by the low excretion of free stilboestrol following the administration of its glucuronide (Wilder Smith & Williams, 1948), and is probably not sufficient to account for the biological activity shown. From evidence so far available it is not, however, possible to formulate the factors to which the lowered potency of the glucuronides is due.

SUMMARY

The isolation and purification of the monoglucuronides of stilboestrol, hexoestrol and dienoestrol, and their chemical and oestrogenic properties, are described.

We are indebted to the Council of the Middlesex Hospital Medical School for the provision of laboratory facilities for this work, to Dr A. C. Bottomley for help with the statistical side of the bioassays and to Mrs D. Culliford and Mr B. D. Shepherd for technical assistance and to the Medical Research Council for a grant.

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The Preparation and Some Properties of Hyaluronic Acid from Human Umbilical Cord

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Hyaluronic acid has now been known for ten years and in this period the literature about it has become both extensive and inconsistent. A number of substances with varying properties has been isolated by different methods from umbilical cord, skin, vitreous humour, synovial fluid, tumours and haemolytic streptococci. In spite of the differences in their properties, all are called hyaluronic acid if they give more or less viscous solutions and if they are composed for the most part of glucuronic acid and acetylglucosamine. There is no clear evidence whether several substances exist with somewhat similar properties, or whether the observed variations are due to contamination with unrelated material, or to partial destruction of originally homogeneous hyaluronic acid during the isolation.

As a prelude to an investigation of the effect of various agents on the enzymic decomposition of hyaluronic acid we have tried to define the conditions under which a product with more clearly defined properties can be prepared. This was necessary because crude preparations differed greatly in the rates at which they lost viscosity with testis hyaluronidase. Contamination of the preparation by substances activating or inhibiting the enzyme is the most obvious explanation of this phenomenon.

The elementary composition of a hyaluronic acid preparation is of little value as a criterion of purity. The likely contaminants have carbon and hydrogen contents similar to those expected for hyaluronic acid. The nitrogen content is a good index of heavy contamination with protein, but no great confidence should be placed on a 'correct' nitrogen value, for this is easily attained by the simultaneous presence of comparable amounts of protein and a nitrogen-free polysaccharide. There is substantial agreement that hyaluronic acid contains neither phosphorus nor sulphur but freedom from sulphur has not often been achieved in making preparations of hyaluronic acid.

Apart from proximate analysis, hyaluronic acid can be examined for its constituents: glucosamine, glucuronic acid and acetic acid; and the equivalent weight can be determined by titration with alkali. These measurements have not often been made on the preparations used as substrate in studies on hyaluronidase. Where they have been made considerable variation is apparent, especially in the case of determinations of the equivalent weight. This value should be 379 for the free acid, on the assumption that the molecule is so large that the ends may be neglected, but Meyer, Smyth & Dawson (1939) and Meyer & Chaffee (1940) have given a series of values ranging from 400 to 640, and our own values are within this range. It seems likely that the carboxyl group in at least part of the glucuronic acid is not free. The existence of this type of linkage robs the equivalent weight of some of its value as a criterion of purity.

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Viscosity has been widely used as an index of purity and of the extent to which degradation of the hyaluronic acid has been avoided during the preparation. This is legitimate if we assume that the native acid, in all the various sites where it occurs, has the same original viscosity, for many treatments are known which reduce the viscosity but none that increase it.

In Table 1 published values for the viscosity of preparations of hyaluronic acid are collected. It is clear that there is no uniformity in these values, and it is interesting to note that some of these preparations have been described by their producers as undegraded although the viscosity comes low in the list. For comparison the values for fractions made by the methods to be described in this paper are included in the same table.

Table 1. *Comparison of the viscosities of various hyaluronic acid preparations*

Source	Ionic environment	Temp. (°)	Concentration (g./l.)	Relative viscosity	Relative viscosity reduced to 1 g./l.	Reference
Cords	0·15 M-NaCl	20	2·5	9·3	4·3	Meyer, Smyth & Dawson (1939)
Cords	0·067 M-Phosphate buffer, pH 7	20	1·0	4·3	4·3	Blix & Snellman (1945)
Streptococci	0·15 M-NaCl	21	1·0	3·8	3·8	Seastone (1939)
Cords	0·1 M-Citrate-phosphate buffer, pH 7	30	3·8	10·0	3·4	Hale (1944)
Synovial fluid	0·067 M-Phosphate buffer, pH 7	20	1·0	2·55	2·55	Blix & Snellman (1945)
Cords	0·66 M-NaCl	34	1·0	2·0	2·0	McClean & Hale (1941)
Vitreous humour	0·15 M-NaCl	21	1·0	1·9	1·9	Seastone (1939)
Vitreous humour	0·067 M-Phosphate buffer	20	1·0	1·85	1·85	Blix & Snellman (1945)
Cords	Borate, chloride, phosphate, acetate mixture; 0·34 M in all, pH 7	25	2·3	2·0	1·4	Haas (1946)
Pleural exudate	0·15 M-NaCl, neutral	20	1·8	1·54	1·3	Meyer & Chaffee (1940)
Vitreous humour	0·5 M-NaCl	25	2·5	1·4	1·16	Madinaveitia & Quibell (1940)
Cords	0·083 M-NaCl, 0·083 M-citrate buffer, pH 4·5	25	16·5	2·6	1·1	Madinaveitia & Stacey (1944)
Cords	0·05 M-NaCl, 0·05 M-phosphate buffer, pH 7	25	1·0	8·2	8·2	This paper

The viscosities given in Table 1 were measured at different temperatures, at different concentrations, and in different ionic environments; part of the variation is due to these factors. At low salt concentrations the viscosity is high and it is greatly affected by slight variation in the salt concentration, but in the range in which most of the values fall the effect of changes in salt concentration is small. It is well known that the increment in the viscosity of a salt solution due to the presence of hyaluronic acid is not proportional to the concentration of hyaluronic acid but that the curve steepens as the concentration increases. These two factors have been disregarded in compiling the last column in Table 1 so that an approximate comparison of the various preparations can be made. In each case a value for the relative viscosity* at the standard concentration of 1 g./l. has been derived from the relation

$$1+\frac{\text{relative viscosity}-1}{\text{conc. at which viscosity was measured}}.$$

This approximation tends to exaggerate the viscosities of those products that were measured at the higher concentrations, but no better method of getting comparative figures seems to be available. With most of these preparations the curve relating relative viscosity to concentration has not been given, and Blix & Snellman (1945) have found that its slope varies from preparation to preparation.

The technique of fractionation used in this work was tried in order to test the basic idea that 'hyaluronic acid' is a name for a family of substances differing slightly in architecture and greatly in particle size. It would seem that the other workers in the field have tried to remove as much protein as possible and have then precipitated all the more or less viscous material in one fraction in spite of its apparent heterogeneity. In this work the appearance of viscous material in several fractions has not been looked on as a disadvantage; the fractions differ in their properties so much that nothing would be gained by mixing them. There is no satisfactory method of separating a mixture of protein and hyaluronic acid in one operation when the two are present in similar amounts. Digestion with commercial trypsin is not permissible if one intends to use the hyaluronic acid for studies on hyaluronidase because the protease preparations we have used inactivate hyaluronidase; there can be no certainty that separation of the inactivator from the hyaluronic acid has been complete. Several of the preparations referred to in Table 1 were made by removing the protein by shaking with chloroform and amyl or butyl alcohol. This process is very satisfactory for the removal of small amounts of protein in the final stages of a preparation, but the operations are tedious if used at the beginning. We prefer to precipitate most of the protein along with only a small part of the hyaluronic acid as a mucin clot and then to isolate the main part of the hyaluronic acid by the methods to be described.

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* The terminology used in recent papers has not always been consistent. Here relative viscosity means the ratio of flow time of the test solution to the flow time of a solution with the same salt concentration. Relative viscosity − 1 is a measure of the increase in viscosity due to the hyaluronic acid. It is sometimes called the specific viscosity, but we prefer to call it the viscosity increment.

EXPERIMENTAL

Preparation of hyaluronic acid

Human umbilical cords have been used as starting material in all our work. They are washed carefully and then preserved in acetone. After lying for 1–6 weeks in acetone they are cut up into pieces not more than 1 cm. long and extracted for a few more days with fresh acetone. The acetone is then drained off as completely as possible and the cords are extracted with successive lots of water, using about four times the cords' wet weight of water for each extraction and leaving each one for at least 2 hr. The first two extracts contain little but acetone and may be thrown away, but the later extracts bring out some hyaluronic acid accompanied by much protein. These extracts therefore give a mucin clot on acidification; they are combined and extraction is continued until the amount of clot is small. Five to eight extractions may be necessary. The combined extracts are taken to pH 3 and the clot collected so that it can be worked up for hyaluronic acid along with other fractions grossly contaminated with protein.

The residue is passed through a power-driven meat grinder with $\frac{1}{8}$ in. holes in the plate. To reduce the viscosity the ground material is suspended in 3 vol. of approx. 0·1 M-NaCl, and after some hours it is poured into a cloth and the fluid is expressed by hand. The extraction is repeated twice, and the new NaCl solution is allowed each time to lie in contact with the cord for at least 12 hr. If this work is not carried out in a cold room the fluids should be kept saturated with chloroform at all times. For each litre of the combined extracts 20 ml. of approx. 5 N-HCl are added with thorough stirring. If the initial washing of the cords was adequate there will be only a small precipitate; this is stirred gently and intermittently during some hours, until it coalesces into a string which is then removed and added to the mucin clot fraction.

Hyaluronic acid can be precipitated from this fluid in several different ways. The conventional methods of precipitation with several volumes of ethanol or acetone are inconvenient because of the bulk of the fluid involved. 100 g. dry weight of washed cords (about 40 cords) give 6 l. of extract at this stage. Two alternative precipitation procedures are described.

(*a*) $(NH_4)_2SO_4$ (300 g.) are added to each litre of the clear acid fluid. Purified hyaluronic acid is not precipitated by $(NH_4)_2SO_4$, but the residual protein in the solution precipitates and brings out some hyaluronic acid with it. The precipitate that separates entangles air forced out of the solution by the salt; this is convenient for it is easier to allow the mixture to stand till all the solid has collected at the top, then to siphon off the subnatant fluid and compact the solid scum by centrifuging, than to de-aerate and centrifuge down the precipitate. To each litre of fluid 50 ml. of pyridine are added and the mixture is shaken vigorously by hand; some of the pyridine remains undissolved in the $(NH_4)_2SO_4$ solution and a little solid separates at the interface. The mixture is allowed to settle, the clear fluid is siphoned off and the interfacial material is compacted by centrifuging. This centrifuging should be done in a covered tube so that the pyridine does not evaporate, because the pellicle remains insoluble only if some pyridine phase is present. The pellicle generally contains little hyaluronic acid. A further 250 g. of $(NH_4)_2SO_4$ are now added. This forces more pyridine out of solution and it brings with it hyaluronic acid which stays at the interface between the two liquids. On centrifuging in closed vessels, it becomes a compact coherent sheet that can be lifted out of the centrifuge tube with a pair of forceps. Approximately equal amounts of hyaluronic acid are present in the original $(NH_4)_2SO_4$ precipitate and in the second pyridine pellicle, but the former may contain as much as 20% of protein and the latter only 5% judging from the nitrogen content. In each case the protein can be removed by shaking with chloroform and amyl alcohol according to the Sevag technique.

(*b*) A comparable method for separating hyaluronic acid from the acid cord extract involves precipitation with acid ethanol in the presence of $(NH_4)_2SO_4$. It offers no advantages over the method already described and involves handling larger volumes of fluid, but there may be other sources of hyaluronic acid with which it might have advantages. Ethanol (1·33 vol.) is added to the acid fluid; a fine gelatinous precipitate separates and this is entangled with air bubbles. The whole precipitate may be sufficiently coherent to float up in 1 hr., thus enabling one to siphon away most of the fluid. If it fails to do this, the air is removed from the precipitate by exposure to vacuum for a few minutes. The precipitate will now sink and can be centrifuged down. This precipitate is suspended evenly in water and reprecipitated by addition of 1·33 vol. of ethanol; on dialysis it goes into solution slowly as the ethanol is removed. Solid $(NH_4)_2SO_4$ is added to the ethanolic supernatant fluid with vigorous mechanical stirring. It dissolves slowly and about 200 g. are needed to saturate 1 l. On standing, the system separates into two layers; the lower contains much $(NH_4)_2SO_4$ and little ethanol and the upper little $(NH_4)_2SO_4$ and much ethanol. As a rule a precipitate which is relatively uncontaminated hyaluronic acid collects on the interface. When separation has proceeded so far that both liquid layers are clear, as much of each as possible is siphoned off and the interfacial material collected by packing it into a coherent felt by centrifuging. It packs very tightly when centrifuged and should be diluted with water before dialysis, for otherwise a rigid jelly will be formed that dialyzes slowly. The proportion in which these two fractions appear varies from preparation to preparation; there is always an ethanol precipitate, but sometimes little or no material separates on the addition of $(NH_4)_2SO_4$. Residual protein is removed from these fractions, as from the pyridine fractions, by the Sevag technique. For effective removal of protein from these viscous solutions, the concentration must be kept low. Solutions of crude hyaluronic acid containing less than 2 g./l. at pH 4 in the presence of 0·1–0·5 M-salt are shaken briskly by hand with a mixture of one part chloroform to two of amyl alcohol, and then centrifuged. The aqueous solution is shaken again, this time mechanically, with fresh solvent. As a rule little or no emulsion is made on this second shaking. After acidification with HCl the fluid can be either dialyzed directly or precipitated with 1·33 vol. of ethanol and then dialyzed in a smaller volume of water. In the latter case precipitation will be incomplete, but this is an advantage because the unprecipitated material has a lower viscosity than that precipitated. It can be recovered as an interfacial precipitate by adding $(NH_4)_2SO_4$.

The yield of purified material, by either of the two methods, is 1–2 % of the dry weight of the cords. The cord residue and the mucin clots that have been precipitated by adding acid to the extracts contain more hyaluronic acid, and this can be recovered, in a less viscous form, by digestion with proteolytic enzymes. The cord residue, of a consistency such that little more can be squeezed out of it by hand, is suspended evenly in about 5 vol. of 0·01 N-HCl containing 0·5 g./l. pepsin. The pH is readjusted to 2 if it is significantly higher than this, and the mixture is incubated with toluene for 24 hr. at 37°. The greater part dissolves leaving a pink solution and a curd. These are easily separated by filtration on a coarse paper at room temperature. Sufficient gelatin is liberated into the fluid to

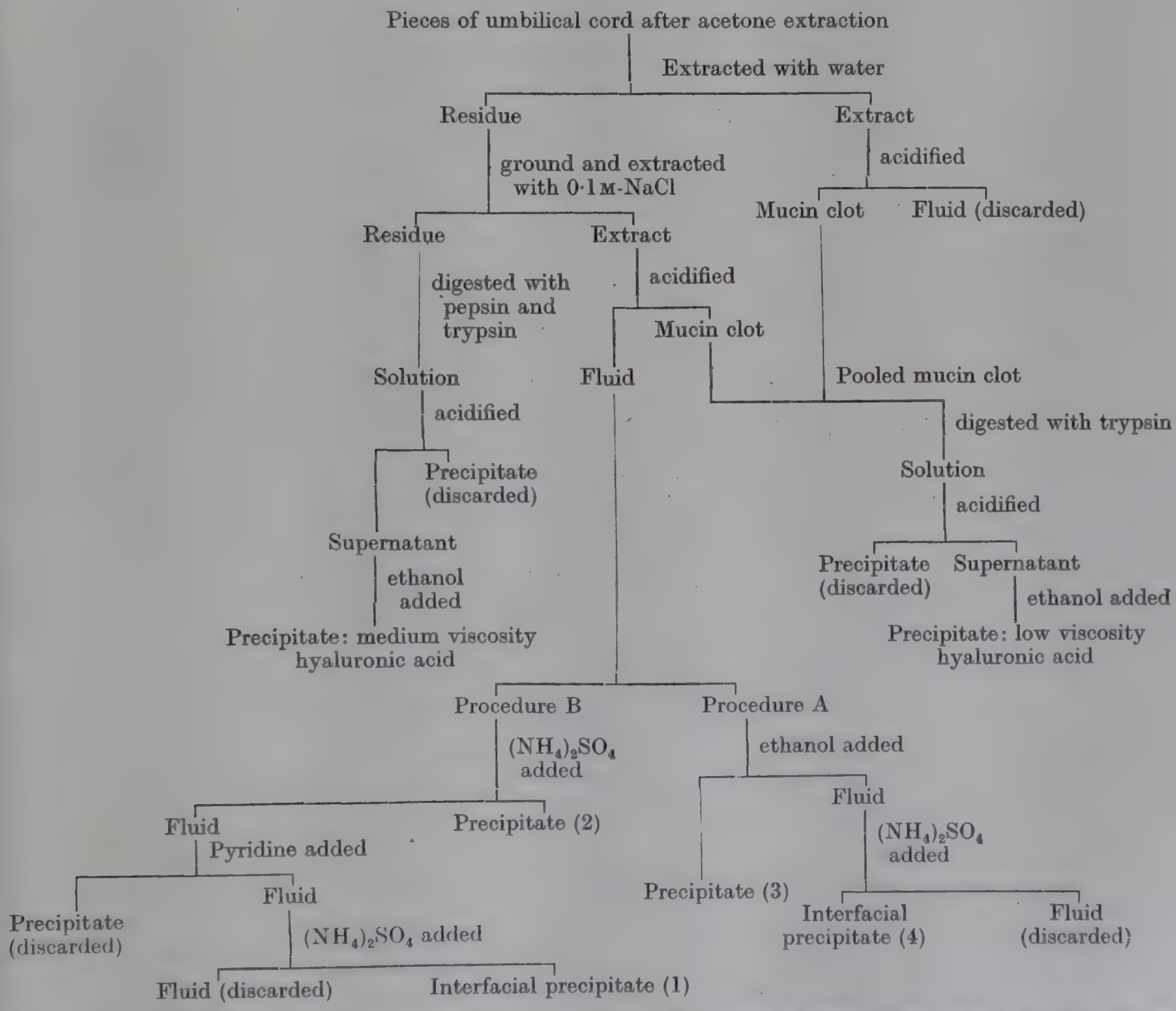
Pieces of umbilical cord after acetone extraction
Extracted with water
Residue
Extract
ground and extracted with 0·1 M-NaCl
acidified
Mucin clot
Fluid (discarded)
Residue
Extract
digested with pepsin and trypsin
acidified
Mucin clot
Solution
Fluid
Pooled mucin clot
acidified
digested with trypsin
Precipitate (discarded)
Solution
acidified
Supernatant
ethanol added
Precipitate (discarded)
Supernatant
ethanol added
Precipitate: medium viscosity hyaluronic acid
Precipitate: low viscosity hyaluronic acid
Procedure B
Procedure A
$(NH_4)_2SO_4$ added
ethanol added
Fluid
Fluid
Precipitate (2)
Pyridine added
$(NH_4)_2SO_4$ added
Fluid
Precipitate (3)
Precipitate (discarded)
$(NH_4)_2SO_4$ added
Interfacial precipitate (4)
Fluid (discarded)
Fluid (discarded)
Interfacial precipitate (1)

Fig. 1. Processes used in the extraction of hyaluronic acid in different forms. Precipitates (1), (2), (3) and (4) are shaken with chloroform and amyl alcohol to give hyaluronic acid fractions with variable but high viscosities.

cause gelatinization if it is cooled. This pepsin extract gives an ethanol precipitate and an interfacial precipitate in much the same way as the extract from the minced cords, but each has a high nitrogen content. It is preferable therefore to neutralize the pepsin extract and digest it with trypsin to destroy the protein that it contains. The cord residue is also digested with trypsin and the two extracts are combined. These digestions are carried out at 37° in the presence of toluene with 2 g./l. commercial trypsin for 24 hr. at pH 7·6. When these trypsin-digested fluids are put through the acid-ethanol-$(NH_4)_2SO_4$ fractionation, nothing precipitates with the conc. HCl and very little interfacial material separates. What there is, therefore, is added to the ethanol precipitate and this precipitate is dissolved, reprecipitated, and dialyzed.

The mucin clot can be partially fractionated by precipitation with ethanolic potassium acetate in alkaline solution as in McClean's (1943) method of purification, but it is generally more satisfactory to incubate it with trypsin and then work up the hyaluronic acid by ethanol precipitation in the manner just outlined for the pepsin extract of the cord residue. The yields of these two products are variable, but that from the pepsin extract may be as high as 4%, and that from the mucin clots 1%, of the dry weight of the cords. It is clear, therefore, that our methods for the preparation of the viscous fractions of hyaluronic acid lead to incomplete extraction in the first place and to significant losses during the purification. This state of affairs seems to be unavoidable if viscous preparations are desired. Material from the pepsin extract has only half the viscosity increment of the viscous fractions and that from the mucin clots is even less viscous. Slightly more viscous products can be made from these by precipitating a series of fractions with ethanol or ethanolic potassium acetate, but no product has been made from them at all resembling the viscous fractions. In Fig. 1 an outline is given of the processes used in making these various fractions, and also of the sequence in which they occur.

Properties of preparations of hyaluronic acid

General

Umbilical cords that have been washed with acetone and water and dried contain 2·5–3% of amino sugar estimated as glucosamine by the method of Elson & Morgan (1933). This amount of glucosamine would correspond to 6–8% of hyaluronic acid, which is equal to the total yield that we get. The non-viscous fractions however contain less than the theoretical percentage of glucosamine. Some of the glucosamine is therefore unaccounted for, but it is clear that the greater part of the glucosamine of the cord is present in the form of polysaccharides that can be isolated by the methods described here.

Preparations made from the acid-soluble part of the original cord extract are all clear and colourless, those made by digesting the mucin clot or the cord residue are yellow or pink. The nitrogen content (Kjeldahl) lies in the range 2·8–4·3%. The phosphorus content is lower than 0·05%, but sulphur contents of 0·5–1% are usual. The form in which this sulphur occurs is not known. Viscous preparations contain between 9 and 11% of acetyl, but preparations made by enzymic digestion of the residues have lower acetyl contents, sometimes as low as 6%. The acetyl content was measured by hydrolyzing a sample for 75 min. at 100° in 2·5N-H_2SO_4, steam distilling in the apparatus described by Markham (1942) and titrating the distillate with N/70 NaOH. There is a similar deficiency in the glucosamine content; non-viscous preparations contain 20–25%, whereas viscous preparations contain 34–38%. These values are in the range found by most other workers. Glucosamine determinations were made by the Elson & Morgan (1933) technique on material that had been hydrolyzed for 6–8 hr. at 100° with 5N-HCl and then evaporated to dryness in a vacuum desiccator over NaOH. These analyses were made on portions of dialyzed solutions, the concentration of which was determined by drying at 100° for 1–2 hr., and cooling in a desiccator. They refer to the free acid and not to a salt.

Viscosity

Viscosity measurements were made in Ostwald viscosimeters with capillaries 9 cm. long and having flow times of about 30 sec. for 4 ml. of water. This volume was used in all the experiments reported here and, except where another temperature is specified, all measurements were made at 25°. Viscosity measurements made in the absence of salt are of little significance; the large influence exerted by low concentrations of salt makes such a measurement an index of the completeness of dialysis rather than of the state of aggregation of the hyaluronic acid. From a consideration of the results of Madinaveitia & Quibell (1940) and of our own results we have adopted 0·05M-NaCl and 0·05M-phosphate buffer at pH 7·0 as the standard ionic environment for measurements. Small variations in salt concentration in this region have little effect on the viscosity. Much of the work on hyaluronic acid has been done at pH 7, and we adopted this pH because we intended to use it in subsequent enzyme studies. A more acid pH would be in some ways preferable, for the viscosity of hyaluronic acid in 0·025M-citrate buffer and 0·15M-NaCl, is unaffected by the pH in the range 4·5–7·3. There is a sharp fall in viscosity to about half the maximum between pH 4·5 and 2·6. Above pH 7·3 there is a more gradual fall to 95% of the maximum value at pH 8·5.

Pyridine precipitates, before the removal of residual protein by shaking with chloroform and amyl alcohol, are the most viscous products that we have encountered. Solutions containing 0·3 g./l. have a relative viscosity in salt of 2·2–2·5, but this falls to 1·9–2·2 when the protein is removed. The results of Blix & Snellman (1945) suggest that this may be due to atmospheric oxidation during the shaking; it might be avoided, as they suggest, by shaking in nitrogen but we have not tried this. The three other viscous products, after removal of protein, have relative viscosities of 1·7–2·0 for the 0·3 g./l. solution. In these cases the removal of protein may be accompanied by either a rise or a fall in viscosity. Occasional batches have had viscosities lower than this, and there is a gradual fall in viscosity as solutions age. Products from the cord residue have relative viscosities of 1·3–1·5 at 0·3 g./l. and those from the mucin clot 1·01–1·15. McClean & Hale (1941) and Blix & Snellman (1945) have published data on the non-linearity of the relationship between viscosity and the concentration of hyaluronic acid solutions. Our results are in agreement; with viscous preparations an increase in the concentration from 0·3 to 0·6 g./l. increases the viscosity increment three- to fourfold, and solutions containing 1 g./l. have a relative viscosity of more than 8. With less viscous preparations the relationship is practically linear.

Effects of temperature on the viscosity

Differences in the temperature at which the measurements were made must contribute to the differences in the results collected in Table 1. The flow times of 0·3 g./l. solutions of preparations covering a range of viscosities have been measured at various temperatures in the salt mixture already specified. The flow time of all is reduced by approximately one third between 20 and 37°, but much of this reduction is due to the change in the viscosity of the buffer solution. Over this temperature range the change in viscosity increment is never more than 15 % of the value at 20°. Temperature is clearly not a significant factor in explaining the differences between products made in different laboratories.

Effect of different salts on the viscosity

Madinaveitia & Quibell (1940) and McClean & Hale (1941) have shown that the viscosity of hyaluronic acid depends on the ionic environment. We have tried the effect of several salts on different preparations, and the results shown in Fig. 2 are typical of those given by viscous preparations from which the protein had been removed by the Sevag technique. An abrupt fall in viscosity with traces of salt is followed by a more gradual fall to a minimum viscosity at about 0·5 M. There is then a gradual rise in relative viscosity; the values at 1 M are approximately the same as those at 0·01 M.

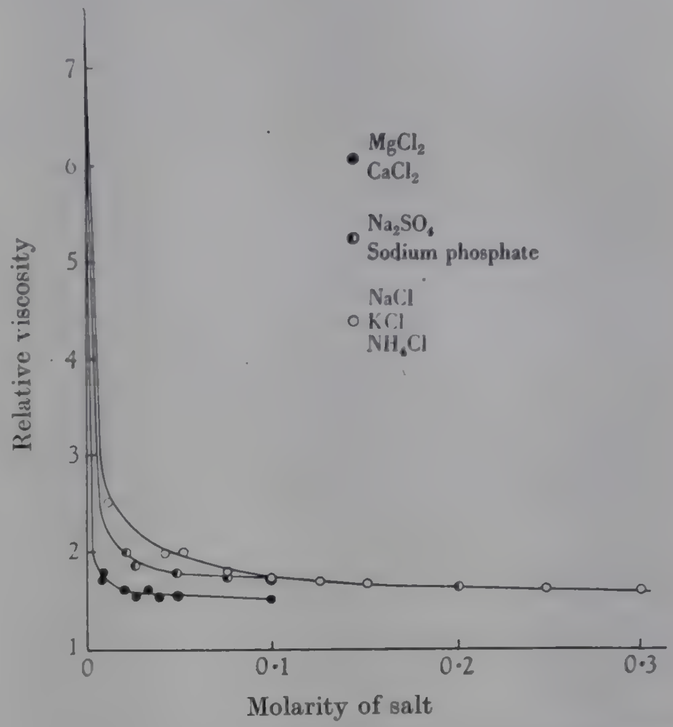

Fig. 2. Change in the viscosity of hyaluronic acid solution with varying concentrations of different salts. Temp. 25°; pH 7; 0·3 g./l. solution of a medium-viscous preparation.

Calcium and magnesium chlorides have clearly a greater effect than the other salts, but no generalizations can be made on the basis of such a small number of salts.

SUMMARY

1. Methods are described for preparing hyaluronic acid from human umbilical cords.

2. By preparing a series of fractions some products have been made with a greater viscosity than any hitherto described.

3. Some data on the chemical and physical properties of these fractions are given.

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The Effects of Serum and of Hyaluronic Acid Derivatives on the Action of Hyaluronidase

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In the preceding paper (Hadidian & Pirie, 1948) the viscosity of hyaluronic acid preparations in a defined environment was used as a criterion in selecting standard preparations. In this paper the loss of viscosity by such a preparation has been used to follow the course of hyaluronidase action. This method has the primary advantage that loss of viscosity appears to be the first change that can be recognized during the enzymic decomposition of hyaluronic acid. Factors influencing this *in vitro* change are more likely, therefore, to affect the action of hyaluronidase *in vivo* than those influencing the later stages of the decomposition of the molecule. It is clear that several enzymes are involved in the hydrolysis of hyaluronic acid to reducing sugars. Thus Hahn (1945) has effected a partial separation of the enzyme responsible for oligosaccharide production from that responsible for monosaccharide production; and Rogers (1946*b*) finds differences between the products of the reaction catalyzed by enzymes from different sources. He interprets this as evidence that the enzymes studied by Hahn are present in different ratios in these sources and points out that it is improbable that either enzyme brings about the reduction in viscosity.

Several inhibitors are known for the action of hyaluronidase on hyaluronic acid, whether this is measured by fall in viscosity, mucin-clot prevention or appearance of acetylglucosamine. McClean & Hale (1941) and Hobby, Dawson, Meyer & Chaffee (1941) found that the sera of animals, that had been injected with hyaluronidase from different sources, inhibited the action of the enzyme used for immunization but did not inhibit the action of other hyaluronidases. Later, however, McClean (1942) mentioned briefly an inhibitor present in normal serum, and this substance has also been investigated by Haas (1946). Heparin, gastric mucin, chondroitin sulphuric acid and partly depolymerized hyaluronic acid also inhibit the enzyme. McClean (1942) suggests that these glucosamine-containing substances act competitively because of their structural similarity to hyaluronic acid. Working on this suggestion we have made some inhibitors by modifying hyaluronic acid. We also record some observations on the inhibitor present in serum.

EXPERIMENTAL

General

Viscosity measurements were made by the technique used in the preceding paper (Hadidian & Pirie, 1948). The time taken for the viscosity to fall half way from the initial to the presumed final value has been taken as a measure of the rate of enzyme action. This measurement of 'half-time' is conventional in studies on this enzyme and has been adopted for convenience, although it is clear that it has no particular merit. It does not, for example, correspond to the loss of viscosity by half of the hyaluronic acid present but to a smaller proportion than this; and to a proportion that varies inversely with the original viscosity of the hyaluronic acid. This is due to the non-linearity of the relation between the concentration and the viscosity of a hyaluronic acid solution, and to the non-linearity of the relation between the axial ratios of elongated particles and the viscosity of their solutions. The enzyme action we are studying can be pictured in two extreme ways. The enzyme may attack a particle of hyaluronic acid and proceed to break linkages in that particle until the products have little or no effect on the viscosity of the solution; the half-time would correspond to a state where less than half the particles have been affected because of the first non-linearity. On the other hand, it may break all the particles to some extent before any one particle has been so far reduced as to have little effect on the viscosity of the solution; because of the second non-linearity the half-time would correspond to the breaking of less than half the relevant linkages.

Some confusion is also introduced into the use of half-time measurements in studying inhibition reactions by the viscosity of the inhibitors. In many cases the viscosity of the inhibitor is unaffected by the enzyme; the final value for the end of the action can therefore be taken as the viscosity of the inhibitor solution in the salt mixture used. In some cases, however, the sum of the increments in relative viscosity, due to hyaluronic acid and inhibitor separately, is less than the observed increments when they are present together. This effect, when it occurs, is small and as long as no significance is attached to differences in half-time less than 30%, it is unlikely to alter the conclusions.

Measurements of the rate of loss of viscosity have been made on an extensive range of hyaluronic acid preparations under identical conditions and with the same enzyme preparation. The half-times have varied by a factor of ten. Part of this variation is due to differences in the initial viscosities of the preparations. If differences in viscosity are due to differences in the average length of the particles in a preparation, the half-time corresponds to the breaking of a smaller number of linkages when the particles are long than when they are short. The generalization that the more viscous the preparation, the shorter the half-time, is not,

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however, found to be invariably true. This has suggested to us that umbilical cord may contain substances that activate or inhibit the action of hyaluronidase on hyaluronic acid, and that these may have been removed to varying degrees during the purification. The fact that viscous preparations are the most uniform in their half-time is in favour of this interpretation. We have tested many of the products discarded during the purification and have found, in agreement with other workers, that fractions of very low viscosity are inhibitors. No fraction giving activation has been consistently preparable.

Preparation of inhibitors

Nitration of hyaluronic acid and substances related to it. Dry hyaluronic acid, with the loose, open structure it has after drying by sublimation, is cooled in a freezing mixture to about −10°. Sixty parts of fuming HNO_3 (sp.gr. 1·59), also cooled to the same temperature, are added and the mixture is maintained at −10° for 15 min. with occasional stirring. All the hyaluronic acid dissolves in a few minutes. Ice (300–400 parts) is now added with assiduous stirring. The temperature falls still further and a white curd separates from a brilliant blue fluid. As soon as most of the ice has melted and all pockets of undiluted HNO_3 in the curd have been broken up with a rod, the mixture is centrifuged for a few minutes and the solid is washed 3–4 times on the centrifuge with ice water. It is kneaded carefully each time to get rid of as much HNO_3 as possible, and the washing is continued until the wash water can be neutralized by a drop or two of 0·1 N-NaOH. The solid is now suspended in distilled water and brought into solution by adding sufficient NaOH to bring the pH to 6–7. This pale yellow, viscous fluid will be referred to as acid-insoluble nitrate.

All the supernatant fluids are mixed and kept cold. They are neutralized with 5 N-NaOH with vigorous stirring and cooling so that the temperature never rises above 20° during the neutralization. The fluid, at pH 6, is concentrated on a hot plate in a draught of air until $NaNO_3$ begins to crystallize out. It is then dialyzed for 10–20 hr. to remove most of the nitrate, concentrated again, and redialyzed. At this stage the solution from 100 mg. of hyaluronic acid has a volume of 2–3 ml. The solution remaining after thorough dialysis will be referred to as acid-soluble nitrate.

The ratio of acid-soluble to acid-insoluble nitrate varies from preparation to preparation. When hyaluronic acid of low viscosity (e.g. with a relative viscosity of 1·4 for a 1 g./l. solution measured in the presence of salt) is used as the starting material there is little acid-insoluble product. When even less viscous fractions are used there is no acid-insoluble material at all. It seemed possible, therefore, that all the acid-soluble material was derived from the less viscous components of an initially non-homogeneous hyaluronic acid. It is unlikely that this is the sole origin; for if the acid-insoluble nitrate is dried by sublimation and nitrated again by the method described, a further quantity of soluble nitrate is formed. Some degradation of the molecule during nitration is to be expected, and this explanation of the origin of some of the soluble nitrate is borne out by the increased proportion of product in that form if the nitration is continued for longer than 15 min. The sum of the weights of nitrate in both forms is about equal to the starting weight of hyaluronic acid and, when highly viscous hyaluronic acid is used, 85% is in the acid-insoluble form.

Nitration always leads to some fall in the viscosity of hyaluronic acid. The extent of this fall is variable, but in general the viscosity increment of the acid-insoluble nitrate is about half that of the parent hyaluronic acid. The acid-soluble nitrate is much less viscous. Nitrated hyaluronic acid, of both types, can be salted out from neutral solution by $(NH_4)_2SO_4$. With the acid-insoluble product, precipitation begins at about one tenth of saturation and is complete at one third of saturation. The more readily precipitated fractions are the more viscous, and they are precipitated fully by lower concentrations of $(NH_4)_2SO_4$ than the less readily precipitated fractions. It is clear, therefore, that the nitrate is a non-homogeneous mixture, but it is not certain whether this reflects an original non-homogeneity of the parent hyaluronic acid or is due to destruction during the nitration. Precipitation of the acid-soluble nitrates and of nitrates derived from hyaluronic acid preparations of very low initial viscosity does not start until one third saturation by $(NH_4)_2SO_4$. Salting out does not therefore seem to be a useful alternative to neutralization, evaporation and dialysis in the preparation of acid-soluble nitrates because, although acid-insoluble nitrate is precipitated by saturated $NaNO_3$, the soluble nitrates are not, and the high nitrate concentration confuses $(NH_4)_2SO_4$ precipitation.

Hyaluronic acid is not salted out by $(NH_4)_2SO_4$ from solutions containing no other component, but, as one of the methods suggested for the purification shows, it can be salted out if pyridine or some forms of protein are also present. The nitrates behave similarly, for, although esterification of the hydroxyl groups has made the nitrates sufficiently hydrophobic to be salted out, they can be salted out at lower concentrations of $(NH_4)_2SO_4$ in the presence of pyridine.

There is no reason to doubt that the hyaluronic acid has nitrated as other polysaccharides do (cf. Speiser & Eddy, 1946); for the dry product burns in a flash and the nitrogen content (Dumas) has risen from 3–4 to 8·6%. No estimate other than the measurement of nitrogen content has been made of the number of nitrate groups introduced. If hyaluronic acid has the structure generally assigned to it, a chain of alternate glucuronic acid and acetylglucosamine residues connected by glucoside linkages, there are four free hydroxyl groups for each repeating disaccharide unit. The sodium salt of the tetranitrate that could be made from such a substance would contain 12% of nitrogen and that of the dinitrate 8·55%. Esterification of the hydroxyl groups in this way does not apparently affect the *N*-acetyl group, for the acetyl content falls from 10–11 to 7–8%. The value to be expected for the dinitrate is 8·7%. Control experiments show that neither nitrous nor nitric acids, in amounts comparable to those present in a hydrolysate, are estimated as volatile acid after hydrolysis and distillation by the procedures used on these samples. On the other hand direct estimation, by the Elson & Morgan (1933) method, on the nitrated material shows an apparent disappearance of the glucosamine. This result is due to the action of the HNO_3 liberated during hydrolysis. The same disappearance can be brought about by hydrolyzing normal hyaluronic acid in the presence of a quarter of its weight of $NaNO_3$, an amount corresponding to four nitrate groups. Furthermore, de-esterification, by the method to be described later, restores the apparent glucosamine content, and hydrolysis in the presence of a reducing agent such as HI protects the glucosamine to a great extent from destruction. Fuming HNO_3 probably acts to some extent as an oxidizing as well as a nitrating agent, but it is clear that no oxidation involving the glucosamine is going on to an extent at all comparable to that found by Skanse & Sandblad (1943) to accompany peroxide oxidation.

Acetylation of hyaluronic acid

Like some other acid polysaccharides (e.g. alginic acid, pectin and agar) hyaluronic acid does not acetylate readily. Acetic anhydride in neutral or alkaline aqueous solution, or in the presence of pyridine under anhydrous conditions, failed to bring about significant acetylation in a few hours at room temperature or on slight warming. Two recent methods are, however, effective; in one the acid is swollen with acetic acid and H_2SO_4 is used as catalyst (Chamberlain, Cunningham & Speakman, 1946), and in the other the acid is swollen with formamide and acetylated with acetic anhydride and pyridine (Carson & Maclay, 1946). The products of these two acetylations differ somewhat in their properties.

(1) Hyaluronic acid is swollen with acetic acid by adding glacial acetic acid in a thin stream to a 3–10 g./l. solution. Mixing is complete until about 2 vol. have been added, after which precipitation begins and is substantially complete with 4 or 5 vol. The mixture is centrifuged and the solid washed with acetic acid and used immediately. This precipitation does not lead to any significant fractionation of the hyaluronic acid, for only about 3% of the starting material is found in the solution when this is evaporated to dryness. This soluble fraction has the normal nitrogen and glucosamine content, but its viscosity is low. The precipitate, on the other hand, if dialyzed immediately, gives a solution with the original viscosity. We do not, therefore, agree with the statement made by many workers on hyaluronic acid that precipitation with acetic acid leads to a fall in viscosity; but our observations are in agreement with those of Meyer, Smyth & Dawson (1939) who prepared a relatively viscous product from synovial fluid by acetic acid precipitation.

The fibrous clot of hyaluronic acid is pressed as free from acetic acid as possible and added to 200 times its weight of a mixture of 1 part of conc. H_2SO_4, 90 parts of acetic anhydride and 120 parts of benzene. There is little or no solution of the hyaluronic acid, and, after a suitable interval, the mixture is centrifuged and the solid suspended in water and dialyzed. If acetylation is allowed to proceed for a few minutes only, the product has an acetyl content of 16–20%; after 40 min. at 22° this rises to 25–33% and is not increased by longer acetylation at that temperature. By following the method of Chamberlain *et al.* (1946) and raising the temperature to 60° for 15 min., after acetylation has proceeded for 1 hr. at 25°, the acetyl content can be raised to 36%. Acetyl determinations were made by the method used in the preceding paper (Hadidian & Pirie, 1948) and include both *O*-acetyl and *N*-acetyl. The theoretical value for the tri-*O*-acetyl derivative of a polysaccharide built up from equal quantities of glucuronic acid and *N*-acetyl glucosamine is 34% and for the tetra-*O*-acetyl derivative 39·4%. It would therefore appear that, as in the case of nitration, esterification remains incomplete. Products that have been acetylated for a few hours only are still soluble in water over the whole pH range, but those that have been acetylated at room temperature for many hours, or have been heated to 60° during acetylation, are not soluble on the acid side of neutrality. There is a progressive fall in viscosity during acetylation. Products acetylated for a few minutes only retain 70–80% of the original viscosity increment, whereas the tri-*O*-acetates retain only 10% or less.

(2) Hyaluronic acid that has been dried by sublimation does not change its appearance when left for a few hours at 20° in formamide, but at 40° it swells somewhat and at 50° turns in 1 hr. to a viscous dough when suspended in ten times its weight of formamide. This dough can be mixed with an equal volume of pyridine by stirring for a few minutes. The plastic, rather than fluid, mixture is now mixed with half its volume of acetic anhydride, which causes an initial shrinkage, but is incorporated into the mixture in a few minutes. As in the other method of acetylation the properties of the final product depend on the duration of acetylation and the temperature at which it proceeds. The mixture is stirred with three times its weight of ice; when it is brought to pH 2 with 5N-HCl a soft curd separates. The fluid is dialyzed. If acetylation has proceeded for 1 hr. at 20° this acid-soluble product has an acetyl content of 14–16%, while after 3 hr. at 30° it rises to 30%. In neither case has it any significant viscosity. The curd is not soluble at neutrality in the absence of salt and is only partly soluble in the presence of salt, but it swells slowly to give a suspension of gelatinous particles, which dissolve at pH 9–10. The solutions have only one fifth of the viscosity of the parent hyaluronic acid. All these acetylated fractions coagulate with strong acid and, like the nitrates, can be precipitated with $(NH_4)_2SO_4$.

De-esterification. The nitrates and acetates of hyaluronic acid can be partly de-esterified without extensive degradation by exposure to alkali in the presence of ethanol. Solutions of either ester containing 3–10 g./l. are mixed with 10 vol. of ethanol. With the esters, unlike with hyaluronic acid itself, no precipitate separates even on the addition of a salt such as potassium acetate. The addition of ethanolic KOH at 22° leads to the appearance of an opalescence, and later, after a time interval depending on the amount of alkali added, of a precipitate. For example, an ethanolic solution containing 0·8 g./l. of a moderately viscous nitrate (rel. visc. 2·3 at 1 g./l. in salt) gave an immediate precipitate with 7 g./l. KOH; but with 2·5 g./l. precipitation took 2 hr., and with 0·7 g./l. precipitation occurred only on the addition of some salt. Similarly with the acetate there is immediate precipitation with 1–2 g./l. KOH, but with less than this amount precipitation is delayed. Under these conditions de-esterification remains incomplete as determined from the nitrogen and acetyl content of these products. The immediate purpose of the work, the making of substances that inhibit hyaluronidase is, however, satisfied; and conditions that would lead to complete de-esterification have not been investigated.

Factors affecting the hyaluronidase-hyaluronic acid reaction

Effect of salt concentration on the rate of reaction. In studying the effect of possible inhibitors and activators on the hyaluronidase-hyaluronic acid reaction, it would first be necessary to establish conditions of salt concentration under which such work can be carried out with the least possible interference from salt effects. Results of Robertson, Ropes & Bauer (1940), McClean & Hale (1941), and

Madinaveitia & Quibell (1941) show that there is an optimum salt concentration for the reduction of the viscosity of hyaluronic acid by hyaluronidase, but the optima they find vary with their different substrate and enzyme preparations. We have tried the effect on this reaction of several salts, previously studied for their viscosity-reducing activity, with results shown in Fig. 1. These reactions were carried out at pH 7·0, using salt solutions buffered with 1% of phosphate or borate buffer. Of necessity, pH control in low salt concentrations was not quite adequate, but frequent checks showed that variation was not more than 0·2 unit; this may produce as much as 20% variation in the rate of reaction. From the results shown and from others on different substrates, three groups are distinguishable: $CaCl_2$ and $MgCl_2$, effective in very low concentrations; NaCl, KCl and NH_4Cl, just as effective but in higher concentrations; Na_2SO_4 and Na_2HPO_4, relatively ineffective. Sodium acetate is intermediate between the chloride and phosphate. Lineweaver & Ballou (1945), in their study of the effect of salt concentration on the activity of pectin esterase, find similar variations in the effectiveness of various salts in activating the enzymic reaction, but with that enzyme there is the same maximum activation at the optimum concentration of each salt. We agree with McClean & Hale (1941) that the chloride ion is an important activating agent. However, the difference in the effectiveness of the chlorides cannot be attributed to Cl^- alone. Expressed as normality of Cl^-, assuming complete ionization, the optima with Mg^{++}, Ca^{++}, Na_4^+, NH^+ and K^+ are at 0·05, 0·06, 0·1 and 0·15 respectively. Furthermore, in the presence of 0·05 M-phosphate, maximum activation is obtained with 0·05 M-NaCl. Two factors seem to be involved; general salt concentration and the presence of an activating ion, such as Cl^-. The pH optimum in the presence of 0·05 M-NaCl and 0·05 M-phosphate is at 6·3; this value is intermediate between the optima of 6·8 and 5·0 found by McClean (1943) in 0·017 M- and 0·167 M-NaCl respectively. Our experiments have been carried out at pH 7 as a compromise between the pH optimum and the pH at which the enzyme and inhibitors are used in *in vivo* tests that will be described elsewhere.

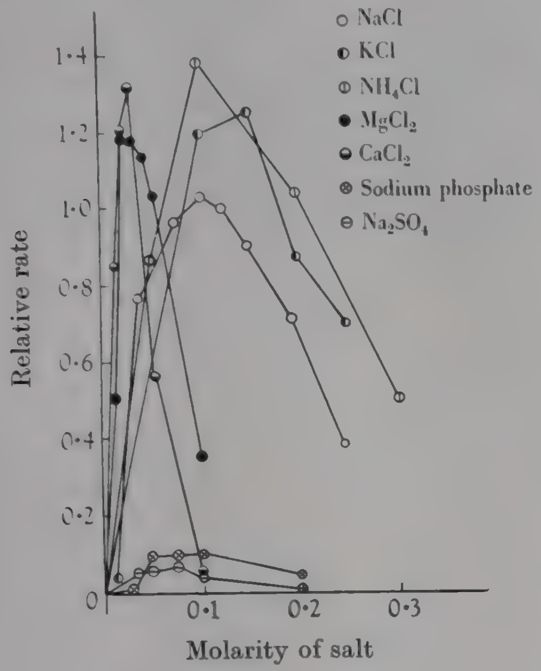

Fig. 1. Variations in the rate of hydrolysis of hyaluronic acid by hyaluronidase with varying concentrations of different salts. Temp. 25°; pH 6·9 ± 0·10; 12·5 mg./l. bull-testis enzyme; 0·3 g./l. solution of a medium viscous hyaluronic acid preparation. In the experiments with NaCl, KCl, NH_4Cl and Na_2SO_4 the pH was maintained with phosphate buffer, and in those with $MgCl_2$ and $CaCl_2$ with borate buffer. In all cases the molar ratio of salt to buffer was 100. In expressing the relative rates, the reciprocal of the half-time in 0·125 M-NaCl is taken as unity.

Estimation of inhibitory activity. Since no method exists for direct estimation of hyaluronidase, the course of the reaction between it and an inhibitor has to be followed by the indirect method of determining the amount of hyaluronidase that is made unavailable for the hyaluronic acid-hyaluronidase reaction in the presence of the inhibitor or after the hyaluronidase has been exposed to the action of the inhibitor for a given period of time. If, then, one works in a region where there is a linear relationship between hyaluronidase concentration and the rate of loss of viscosity by hyaluronic acid, one can justifiably consider the rate as a direct measure of the amount of active hyaluronidase in the system. The range over which such a relation exists varies with different preparations of hyaluronic acid tested at the same concentration. With highly viscous preparations and with the partially purified enzyme preparation that we use, deviation from linearity is slight up to an enzyme concentration of 8 mg./l. With less viscous preparations this range may extend up to 50 mg./l. The following methods are used in estimating inhibitory action: in instances where the length of exposure of the enzyme to the inhibitor is of little or no consequence, the enzyme is introduced into a mixture containing both inhibitor and substrate, and the subsequent viscosity changes followed; where the extent of inhibition is dependent on the duration of exposure of the enzyme to the inhibitor, as in the case of inhibition by serum, the enzyme and the inhibitor are incubated together under the proper salt and pH conditions for a given length of time, then the substrate is introduced into the mixture, and the course of the reaction followed. The extent of inhibition is determined from the ratio of the half-time of the reaction with inhibitor to that without. Thus a threefold inhibition would be obtained if a half-time of 600 sec. is obtained with inhibitor as compared with 200 sec. without. The reciprocal of this ratio is the fraction of the initial enzyme concentration that is active. Partially purified bull-testis enzyme (Schering) was used in all these experiments.

Inhibition by hyaluronic acid esters. Nitrates and acetates that have been made from viscous hyaluronic acid retain much of their initial viscosity. The effect of hyaluronidase on them can therefore be tested in the usual way. The viscosity of nitrates is not reduced by hyaluronidase and the viscosity of acetates often falls at first and then stays constant. We look on acetates behaving in this way as mixtures of material that can be attacked and material that is not attacked and does not inhibit the reaction. The stability of these hyaluronic acid esters towards the enzyme contrasts with the susceptibility to attack of the *p*-aminobenzyl ether (Humphrey, 1943). In Humphrey's preparations, however, less than one ether linkage seems to be present for each glucosamine in the hyaluronic acid, and no indication is given of the extent to which hydrolysis, which was recognized by the appearance of *N*-acetylglucosamine, took place.

Table 1. *Effects of various inhibitors on the hyaluronidase-hyaluronic acid reaction*

Description of inhibitor	Concentration (g./l.)	Inhibition
Viscous hyaluronic acid acetylated by sulphuric method for 60 min.; 33 % acetyl	0·1	1·5
Above product deacetylated for 15 min. with 2 g. KOH/l.; 14 % acetyl	0·1	6
Viscous hyaluronic acid acetylated by sulphuric method for 10 min.; 18 % acetyl	0·1	4
Viscous hyaluronic acid acetylated by formamide method for various times; 15, 25 or 30 % acetyl	0·1	Inactive
Acid-soluble product from nitration of viscous hyaluronic acid	0·01	3·5
Acid-insoluble part of the same nitration	0·1	7·3
Acid-soluble product from nitration of viscous hyaluronic acid	0·03	3·0
Acid-insoluble part of the same nitration	0·03	2·2
Above insoluble product denitrated for 2 hr. with: 0·7 g. KOH/l.	0·005	5
2·5 g. KOH/l.	0·004	7·5
7·5 g. KOH/l.	0·03	2·2
Nitrated non-viscous hyaluronic acid	0·01	5
The same non-viscous hyaluronic acid before nitration	0·1	2
Heparin	0·1	2

In these experiments 2 ml. of a 0·6 g./l. solution of hyaluronic acid in 0·1 M-NaCl and 0·1 M-phosphate buffer pH 7 were mixed with 1 ml. of a solution of the inhibitor at four times the concentration specified in the second column. 1 ml. of a 50 mg./l. solution of hyaluronidase was then added and the flow time in an Ostwald viscosimeter was measured at various intervals. The half-time was found in the usual way, and the amount of inhibition set out in the third column is derived by dividing the half-time found in the presence of the inhibitor by that found in a control experiment with enzyme and substrate alone.

The inhibition is measured by mixing suitable amounts of inhibitor with hyaluronic acid and then adding the enzyme. There is a slight increase in the inhibition if the enzyme and inhibitor are mixed first, but in this instance the extent of inhibition is not increased by allowing the enzyme and ester to be in contact for more than the time necessary for mixing. Some comparable results are collected in Table 1. Values are given for only one concentration of each inhibitor because the inhibition is proportional to the concentration. The table shows that acetates inhibit weakly, or not at all, and the most active are partially acetylated products the preparation of which is a matter of some uncertainty. On partial de-acetylation the inhibition always increases, but conditions that would lead to regularly repeatable results have not been found. The results with nitric esters are more systematic; all of them inhibit. Attempts to get less nitration by using a smaller ratio of nitric acid to hyaluronic acid or by allowing the nitration to proceed for a shorter time have led to less active products. The acid-soluble fractions from the nitration of viscous material and the acid-soluble nitrates of non-viscous material are stronger than the acid insoluble nitrates. By suitably controlled removal of nitrate groups with alkali and ethanol the inhibitory power is substantially increased; but it is clear from the table that the intensity of the alkali treatment is of importance and that too drastic a treatment leads to a weaker inhibitor. For comparison the results of experiments with non-viscous hyaluronic acid and heparin are also included in the table. Many non-viscous preparations of varied origin have been tested and none has been found with a significantly greater inhibitory power than the one cited. Only one sample of heparin was tried; the inhibition found here is comparable to that found by McClean (1942) and Rogers (1946*a*) using different experimental procedures. Our tests were made at 25° with hyaluronic acid having a relative viscosity of 7–8 for a 1 g./l. solution in 0·05 M-NaCl and 0·05 M-phosphate buffer pH 7, although experiments described in the next paragraph show that there is more complete inhibition with smaller concentrations of phosphate. The same sample of dried, partially purified hyaluronidase from bull testes (Schering) was used throughout at 12·5 mg./l. Similar experiments have been made with larger and smaller concentrations of nzyme, at 37° and with samples of hyaluronic acid having a relative viscosity as low as 2 for the 1 g./l. solution. These variations in the conditions have not significantly affected the amount of inhibition observed.

Variations in phosphate and NaCl concentration, however, have a marked effect on inhibition by the esters. Several of these, including an acid-soluble nitrate, an acetate, a denitrated nitrate and a de-acetylated acetate, were tested in 0·05M-NaCl with phosphate concentrations varying between 0·013 and 0·24M; and in 0·05M-phosphate with NaCl concentration varying within the same range. Within this range there is an increasing inhibitory activity with decreasing salt concentration. None shows a significant inhibitory activity in 0·05M-NaCl and 0·24M-phosphate and all show maximum activity in 0·05M-NaCl and 0·013M-phosphate; but they all follow different courses between these extremes. Products with identical inhibitory activity under our standard testing conditions may differ by a factor of 2, at lower phosphate concentrations. Thus the figures given in Table 1 would not necessarily indicate the relative effectiveness of these substances under physiological conditions.

For convenience of writing, the operations that have been performed on hyaluronic acid in making these inhibitors have been called acetylation, nitration and de-esterification. It is certain that the bulk of the material is undergoing these changes, for the observed acetyl content can be increased and diminished as can the Dumas nitrogen content and the inflammability of the nitrates. It is less certain that these are the changes primarily responsible for the inhibition. All of them are associated with changes in viscosity and so presumably in particle size, and it is possible that this is the significant change rather than the extent of esterification. The difficulty found in getting reproducible results from experiments on acetylation and de-acetylation slightly favours this interpretation. On the other hand, the stability of these inhibitors in the presence of hyaluronidase suggests that a graded breakdown of the molecule is not alone sufficient to make a good inhibitor. Furthermore, our attempts to degrade the molecule by other means, e.g. acid, alkali and partial enzyme digestion, have resulted in only feebly active products. It is probable that inhibitors have both an optimum particle size and an optimum degree of substitution. The high activity found with nitrated non-viscous products suggests that this size may be fairly small; but active material does not diffuse through cellophan.

Serum as an inhibitor. McClean (1942), in the course of studying the *in vivo* decapsulation of streptococci, discovered a factor in the sera of several species which inhibits enzyme from different sources, and noted that they varied greatly in their inhibitory activity. He considered the possibility that this inhibition is due to the presence in the serum of a polysaccharide which acts by competing with the substrate for the enzyme. Haas (1946), on the basis of some by no means conclusive evidence, has claimed that this inhibitory action of serum is due to the presence in it of an enzyme which inactivates hyaluronidase.

The inhibition of hyaluronidase by serum at 25° proceeds at a measurable rate, as shown by Haas (1946). The method used for its study by Haas, and with some modification by us, has been to expose the enzyme to the action of serum for a given length of time, then mix it with hyaluronic acid solution containing enough phosphate buffer to obtain a final concentration of 0·05–0·1M-phosphate. The validity of this method rests upon the fact, discovered by Haas and confirmed by us, that in the presence of such concentrations of phosphate (the exact concentration varies with different serum samples) the activity of hyaluronidase in reducing the viscosity of hyaluronic acid is unaltered by incubation with serum. One cannot, however, assume on such evidence that interaction between hyaluronidase and the serum factor ceases in the presence of phosphate.

If one introduces phosphate into a mixture of hyaluronidase and serum that has been incubated for a time sufficient to inactivate a part of the hyaluronidase, and then removes samples at various intervals for testing their hyaluronidase content, one finds that in low phosphate concentrations (0·01–0·05M) the inhibition of hyaluronidase will continue, and in high phosphate concentrations (0·05–0·1M) it will be reversed. However, at about 0·05M the rate of change in either direction is very slow. Since the process of estimating the hyaluronidase after the inactivation reaction seldom lasts longer than 30 min., this method of studying the inhibitory action of serum seems valid so long as significance is not attached to small changes in activity.

Like the reaction between hyaluronidase and hyaluronic acid, the reaction of dialyzed serum with hyaluronidase does not proceed in the absence of salt. It is activated by small amounts, with an optimum between 0·03 and 0·1M-NaCl. The exact optimum varies somewhat with the amount of serum used, but variations within the range specified are slight. In 0·2M-NaCl the inactivation of hyaluronidase by serum at the end of 10 min. of incubation is about one fifth of the maximum. Phosphate will activate this reaction to a lesser extent. However, it is much more effective relatively in this reaction than in the hyaluronidase-hyaluronic acid reaction. In the presence of 0·025M-phosphate the inhibition attains 75 % of the maximum obtainable in NaCl. With higher concentrations of phosphate, as of NaCl, the serum-hyaluronidase reaction is apparently inhibited, 0·075 M-phosphate giving full inhibition. The serum used in these experiments was dialyzed for 4–5 days against several changes of distilled water. For the most part we have used pig serum in this study, but most of the experiments described have been repeated with human, rabbit, chicken or bovine serum. In view of the statement of Haas (1946) that serum obtained by clotting is less active than that obtained from blood defibrinated by stirring, all sera used were obtained in the latter manner. However, in subsequent experiments with sera obtained by clotting and by stirring from the same specimen of blood, we have been repeatedly unable to demonstrate under optimal salt conditions any significant difference between the two.

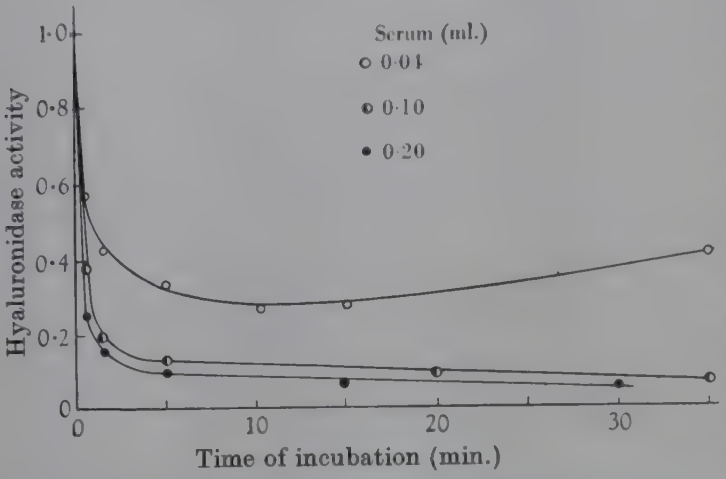

Fig. 2. Rate of inactivation of hyaluronidase by varying amounts of serum. 0·04, 0·1 and 0·2 ml. dialyzed pig serum, pH 7; incubation at 25° with 50 μg. of testis enzyme in 2 ml. of 0·05 N-NaCl containing 0·3 ml. borate buffer pH 6·9. 2 ml. of 0·6 g./l. solution of a high viscosity hyaluronic acid in 0·1 M-phosphate buffer and 0·05 M-NaCl added to the serum-hyaluronidase mixture at the end of incubation and the residual hyaluronidase activity determined viscosimetrically. Hyaluronidase activity $=\frac{\text{half-time without inhibition}}{\text{half-time with inhibition}}$. Owing to the non-linearity of the relationship between enzyme concentration and the rate of hydrolysis of this particular hyaluronic acid preparation the actual amount of hyaluronidase, expressed as fraction of the original amount remaining active after incubation is about a third more than the activity indicated.

As a result of these findings, the following procedure was adopted for testing the inhibitory activity of serum, with such modifications as were required by varying conditions. The enzyme (50 μg.) is incubated with 0·05–0·3 ml. of dialyzed, neutralized serum for 10 min. in 2 ml. of 0·1 M-NaCl buffered with 0·2 ml. of 0·2 M-borate buffer at pH 6·9. To this is then added 2 ml. of hyaluronic acid solution, 0·6 g./l. in 0·1 M-phosphate, and the course of the depolymerization of hyaluronic acid followed viscosimetrically.

Having established beforehand the range of enzyme concentration over which the relationship between hyaluronidase concentration and the rate of loss of viscosity by the hyaluronic acid preparation to be used does not deviate too markedly from linearity, one can follow the course of apparent inactivation of the enzyme by the serum. At 25° with 50 μg. of enzyme and 0·10 ml. of dialyzed pig serum in 2 ml. of buffered 0·05 M-NaCl, only about 50 % of the enzyme remains active 30 sec. after mixing, and 25 % after 1 min. (Fig. 2). Maximum inhibition is reached within 10–500 min. depending upon the serum concentration. With high concentrations of serum for the next 5–10 hr. no apparent change occurs, but eventually, in every instance the hyaluronidase activity begins to return (Fig. 3).

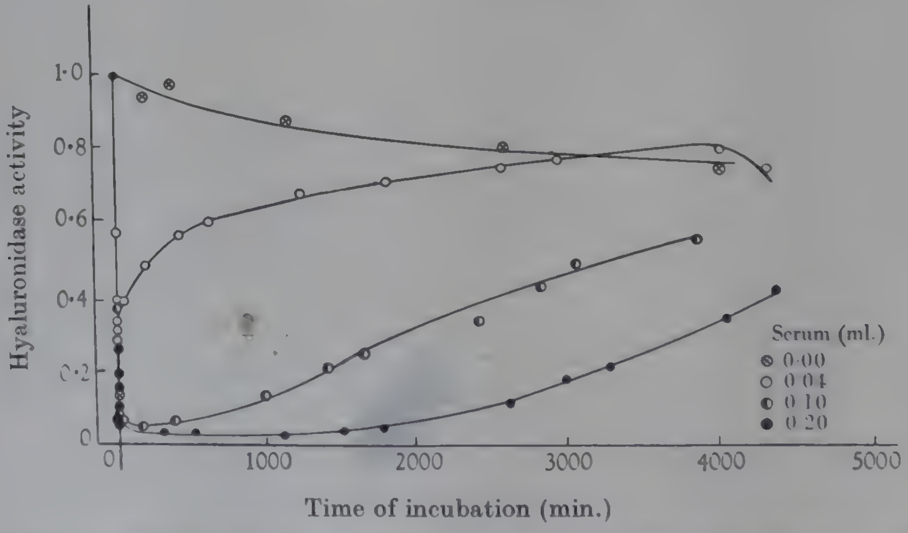

Fig. 3. Recovery of hyaluronidase activity after its inhibition by varying amounts of serum. Conditions of experiment are the same as specified under Fig. 2, where the course of the reaction for the first 35 min. is shown in detail. Serum incubated under the same conditions, but without hyaluronidase, had lost none of its inhibitory activity at the end of the experiment when tested on a fresh enzyme solution.

When there is no excess of serum, with 0·04 ml., recovery of hyaluronidase activity immediately follows its maximum inhibition. When there is an excess, apparently a condition of equilibrium is reached and is maintained as long as there is an excess of the serum factor available. The recovery process in every instance is slow, and is complete within 72 hr. only with low concentrations of serum. A hyaluronidase solution kept under the same temperature, salt and pH conditions may lose a quarter of its activity during this period. Serum kept under identical conditions loses very little, if any, of its original activity. In repeated experiments, the final hyaluronidase activity was never more than that at the beginning of the experiment, thus making it unlikely that this phenomenon is due to the production of hyaluronidase by contaminating bacteria. Experiments were also carried out in the presence of 0·05 M-NaF and 0·05 M-benzoate to prevent any possibility of bacterial contamination. Both interfere to some extent with hyaluronidase activity but the reappearance of hyaluronidase after inhibition can be demonstrated in their presence.

In these experiments inactivation and re-activation proceed in a solution that is not otherwise altered. By changing the environment the re-activation process can be hastened. Dilution of the mixture without change in salt concentration causes a 15 % increase in the rate for fourfold dilution. The same dilution doubles the rate of re-activation if it is made with NaCl containing 0·03 M-phosphate and quadruples it if made with NaCl containing 0·1 M-phosphate.

We find the reaction between serum and hyaluronidase much faster than is manifest from the data of Haas (1946). This in part is due to the fact that Haas uses no salt in his reaction mixture except borate buffer and whatever salt there is present in the serum, in part to the fact that he has no determinations at less than 5 min., and in part to his presentation of the course of the reaction as an increase in the 'activity' of serum. Thus, according to his data, 0·1 ml. chicken serum has an 'activity' of 2 at the end of 5 min., and 4 at 20 min. The former corresponds to an inhibition of 65 % of the hyaluronidase; and the latter to 80 %, i.e. only an additional 15 % of the original hyaluronidase is inhibited between 5 and 20 min. How much of the 65 % inhibition at the end of 5 min. takes place in the first few seconds there is no way of determining from Haas's data, but from our own data it is evident that the reaction is slowed abruptly after about 1 min. when an excess of serum is present.

The inhibition of hyaluronidase by derivatives of hyaluronic acid resembles the serum inhibition, in a general way, in its dependence on the ionic environment, although the serum inhibition is suppressed by concentrations of phosphate at which all esters tested have retained some activity. The actions differ in two significant respects; ester inhibition reaches its maximum more quickly than serum inhibition and there is no recovery on prolonged incubation. It is obvious that the hyaluronic acid esters cannot be called enzymes, but the terminology most suitable for the serum inhibitor is uncertain. Haas (1946) has called it an enzyme because the reaction proceeds at a measurable rate and because the inhibitor is destroyed at 55°, but these properties, which we confirm, are not exclusively the properties of enzymes and the fact that the inhibited hyaluronidase can recover its activity is a strong argument against the applicability of the word enzyme to the inhibitor. Essentially, an enzyme is a substance that combines with its substrate in such a way that, when dissociation occurs, the substrate is altered; the serum inhibitor combines with hyaluronidase and dissociates from it without affecting the only property of hyaluronidase of which anything is known. Under these circumstances the analogy would seem to be with the antigen : antibody and toxin : antitoxin reactions rather than with the enzyme : substrate reactions. The indiffusibility of the inhibitor and its inactivation by heating suggest that it is, or that it is associated with, a protein and this is borne out by McClean's observation, which we confirm, that it precipitates in the globulin fraction of serum. The destruction of the inhibitor on incubation with a hyaluronidase preparation could be due to a protease present in the enzyme, but it is also possible that it is another manifestation of hyaluronidase activity. On this view the inhibitor is a substrate for hyaluronidase on which the enzyme acts slowly; it is this that distinguishes it from the esters for they are apparently not attacked at all by the enzyme. In an attempt to get evidence for this point of view we have estimated the glucosamine in hydrolysates of the ultrafiltrate from dialyzed serum that had been incubated with hyaluronidase for sufficiently long to destroy a part of its inhibitory power. No glucosamine could be detected. This suggests that the inhibitor does not contain glucosamine, that this is not liberated during its destruction, or that the quantity is too small for detection by our methods. There is therefore still no evidence on the nature of the inhibitor or the mechanism of its action.

SUMMARY

1. The effect of various salts on the action of hyaluronidase on hyaluronic acid has been studied viscosimetrically. There is an optimum molarity for each salt, but its value and the rate of the reaction at the optimum vary with different salts.

2. Methods of preparing nitrates and acetates of hyaluronic acid are given. These esters inhibit the

enzyme action and this inhibition is compared with that brought about by a constituent of normal serum.

3. The extent of the inhibition by these agents is dependent on the salt concentration.

4. The serum inhibitor, unlike the inhibitors made by esterifying hyaluronic acid, is destroyed by the testicular hyaluronidase used.

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The Fate of Certain Organic Acids and Amides in the Rabbit

3. HYDROLYSIS OF AMIDES BY ENZYMES *IN VITRO*

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In the first two papers of this series it was shown that the rabbit is able to hydrolyze the carbamyl group in benzamide and phenylacetamide (Bray, Neale & Thorpe, 1946), but that the introduction of a hydroxyl group in the position *para* to the carbamyl renders the latter stable, less than 7% hydrolysis occurring *in vivo* (Bray, Ryman & Thorpe, 1947). Further investigations are in progress of the effect of hydroxyl groups in other positions and also of amino and nitro groups. This paper reports a parallel study in which we have examined the activity of various enzyme preparations, especially those from rabbit liver, in hydrolyzing the carbamyl group.

Similar investigations are those of Gonnermann (1902, 1903), in which the action of several enzyme preparations, including pepsin, trypsin and sheep's liver and kidney, on a number of amides and anilides was studied, of Geddes & Hunter (1928) and of Grassmann & Mayr (1933) who examined the action of yeast asparaginase on several amides. The criterion used by Gonnermann as an indication of hydrolysis was the isolation of the unchanged amide or of products of its hydrolysis, whilst Geddes & Hunter estimated the ammonia liberated. Relevant results from these investigations are summarized in Table 1. Geddes & Hunter claim that asparaginase is present in calf liver and also that yeast asparaginase will hydrolyze glutamine. Glutaminases hydrolyzing this amide but not asparagine have been shown to be present in various tissues, including liver and kidney (Krebs, 1935).

In the present work we have determined quantitatively the degree of hydrolysis by a formol titration method based on that used by Balls & Lineweaver (1939) for assessing the activity of papain with hippurylamide as substrate. We have also compared the stability of the amides used to acid and alkaline hydrolysis.

METHODS

Materials

Amides. The amides which could not be purchased were prepared by the action of aqueous ammonia upon the corresponding acid chloride or upon the methyl ester of the parent acid. Aminobenzamides were prepared by the reduction of the nitrobenzamides with ferrous sulphate (Jacobs & Heidelberger, 1917).

Table 1. *Summary of results of some previous investigations of enzymic hydrolysis of amides*

(Results marked * due to Geddes & Hunter (1928). Results marked † due to Grassmann & Mayr (1933). Unmarked esults due to Gonnermann (1902).

	Amide						
Action of	Formamide	Acetamide	Propionamide	Benzamide	Salicylamide	Asparagine	Glutamine
?epsin	−	−	.	−	−	.	.
Trypsin	−	+	.	−	−	.	.
?tyalin	−	−	.	−	−	.	.
Invertase	−	−	.	−	−	.	.
Maltase	−	−	.	−	−	.	.
Emulsin	−	+	.	−	−	.	.
Sheep's liver	+	+	.	+	.	.	.
Calf's liver	.	.	.	.	.	+*	.
Sheep's kidney	+	−	.	+	+	.	.
Yeast asparaginase	−*	−*†	−*†	.	−*	+*†	+* −†

Sand. British Drug Houses Ltd. acid-washed sand was found to give more satisfactory results than other preparations tried.

Buffers. The majority of the experiments were carried out at pH 7·4 in a phosphate buffer consisting of 0·2 M-Na_2HPO_4 (8 ml.) and 0·2 M-NaH_2PO_4 (2 ml.). Where other buffers were used details are given in the appropriate section.

Preparation of extracts

The rabbit was killed by a sharp blow on the back of the neck and immediately bled. The liver and kidneys were removed and weighed. The organs were then separately ground with sand and mixed with water (1·5 times the original weight). The resulting *brei* was centrifuged and the supernatant liquid used in the experiments, either directly or after dialysis for 24 hr. The extracts were preserved with chloroform.

Method of estimation of amide splitting

Liver or kidney extract (10 ml.) was added to 50 ml. of a 0·0174 M-solution of the amide in the appropriate buffer. The mixture was incubated at 37° in a stoppered flask. At suitable intervals 4 ml. samples were withdrawn, treated with 0·5 ml. neutralized 40 % formaldehyde and 1 ml. 0·1 N-NaOH and centrifuged. Five ml. of the supernatant liquid were titrated against 0·02 N-NaOH using thymol blue, taking the first appearance of blue (pH 9) as end point. Thymol blue was more convenient than phenolphthalein owing to the reddish colour of the digests. With the nitrobenzamides, which are sparingly soluble, 0·00687 M-solutions were used. Complete hydrolysis of 4 ml. digest corresponds to a titre of 2·9 ml. 0·02 N-NaOH. The corresponding titre for nitrobenzamides is 1·14 ml.

Controls used were (i) the mixture as above, but with boiled extract, and (ii) the mixture without the substrate. The titration values of such controls remained steady throughout the experiments except in a very few cases when the titre of the control increased slightly (up to 0·3 ml. 0·02 N-NaOH). The indistinctness of the end point makes it doubtful whether titration differences of less than 0·1 ml. are of any real significance.

Calculation of results

Digests prepared as above using a 0·0174 M-solution of the ammonium salt of the acid instead of the corresponding amide gave results in close agreement with the theoretical values for complete hydrolysis. This theoretical increase in titre was used as the basis for calculation of the degree of hydrolysis.

RESULTS

Experiments with papain

The hydrolysis of benzamide, *p*-hydroxybenzamide and *p*-nitrobenzamide was compared with that of hippurylamide (Balls & Lineweaver, 1939). Digests were made up from 20 ml. of papain solution (3 % papain (British Drug Houses Ltd.) in water) and 50 ml. of the amide (0·0174 M in acetate buffer pH 4·9). A control for each amide consisted of 20 ml. boiled papain solution with 50 ml. of the amide solution. A control of 20 ml. papain solution with 50 ml. buffer pH 4·9 was also included. Typical results are shown in Table 2 and Fig. 1. Complete hydrolysis in this experiment corresponds to an increase in titre of 2·47 ml. 0·02 N-NaOH (0·98 ml. in the case of *p*-nitrobenzamide). *p*-Hydroxybenzamide did not appear to be hydrolyzed at all; the increase in titre of *p*-nitrobenzamide was so small as to be of doubtful significance.

Experiments with liver extracts

Hydrolysis of benzamide. The degree of hydrolysis was determined for each liver extract. Two typical results are shown graphically in Fig. 5. The following figures show the percentage hydrolysis of benzamide effected by 29 different liver extracts in 10 hr. (The figures in parentheses give the percentage hydrolysis at equilibrium.) 38 (47); 20 (58); 11 (44); 35 (44); 20 (32); 19 (44); 19 (46); 9 (34); 27 (77); 15 (53); 14 (25); 4 (40); 17 (52); 38 (60); 16 (37); 35 (38); 44 (47); 32 (44); 39 (62); 32 (45); 33 (38); 27 (35); 44 (55); 25 (52); 26 (49); 15 (51); 14 (40); 15 (33); 22 (32). It can be seen that there is considerable variation. The average values are 24 % for 10 hr. and 45 % at equilibrium.

Table 2. *Hydrolysis of* 0·0174 M-*solutions of hippurylamide, benzamide,* p-*hydroxybenzamide and* p-*nitrobenzamide* (0·00687 M) *by papain at* pH 4·9

Time (hr.) ...	0	0·5	1·0	1·5	2·5	3·75	4·0	5·25	6·75	24	28
	Titres in ml. 0·02 N-NaOH										
Hippurylamide	6·75	7·05	7·2	7·3	7·55	7·7	—	7·75	7·85	8·05	8·1
Hippurylamide control	7·95	—	—	—	—	—	7·6	—	—	7·6	7·65
Benzamide	6·6	6·75	—	6·8	6·85	—	—	6·9	—	6·9	—
Benzamide control	7·7	—	—	—	—	—	7·7	—	—	7·65	—
p-Hydroxybenzamide	8·05	—	—	—	8·0	—	—	7·95	—	8·05	—
p-Hydroxybenzamide control	8·3	—	—	—	—	—	8·35	—	—	8·45	—
p-Nitrobenzamide	6·6	6·7	6·65	—	6·65	—	—	6·75	—	6·75	—
p-Nitrobenzamide control	7·15	—	—	—	—	—	—	—	—	7·05	—
Enzyme + buffer	6·85	6·8	—	6·7	—	7·0	—	6·8	—	6·85	6·85

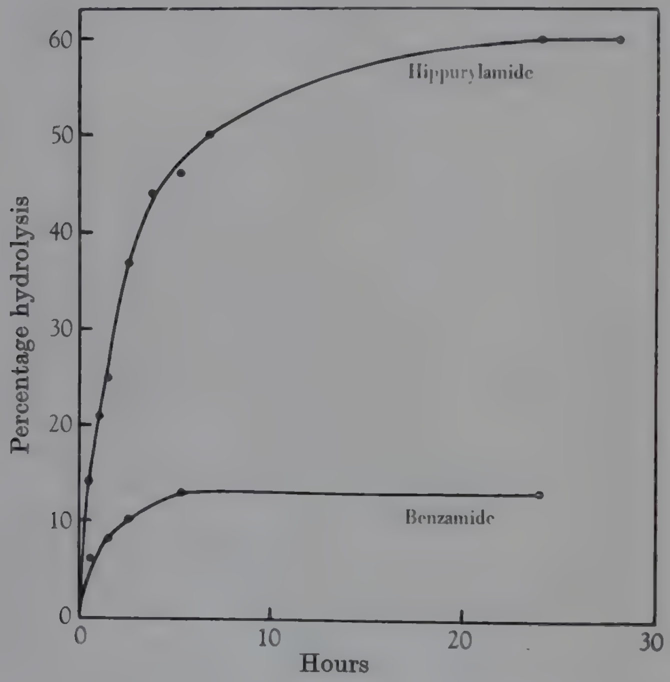

Fig. 1. Hydrolysis of 0·0174 M-hippurylamide and benzamide by papain at pH 4·9.

Effect of pH *on activity of liver extracts.* Several experiments of which the following is typical were carried out in order to determine a suitable pH for the reaction. Benzamide digests were prepared with phosphate buffers of pH ranging from 5·00 to 8·04, the concentration of the amide being 0·210 g./100 ml. To 50 ml. of each were added 10 ml. of liver extract; the usual controls were also prepared. For titrating samples of the digest at pH 5·9 and 6·4 it was convenient to add 2 ml. of 0·1 N-NaOH before titration, while to samples of the digests at pH 6·9 and 7·4, 1 ml. 0·1 N-NaOH was added. Water (1 ml.) was added to the digest at pH 8·04. The results are shown in Fig. 2. It is evident that the pH (7·4) used throughout the main investigations is a suitable one.

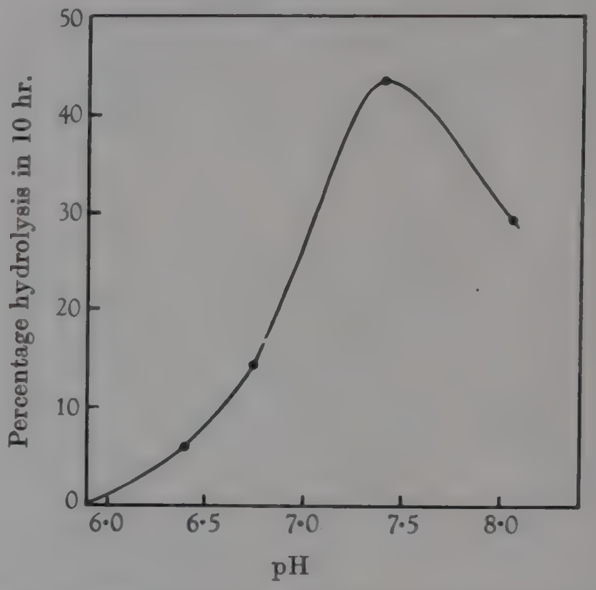

Fig. 2. Effect of pH on hydrolysis of benzamide by rabbit-liver extract.

Concentration of substrate. These experiments were carried out in order to compare the hydrolysis of benzamide and *p*-nitrobenzamide more closely, as in the main experiments the solutions of the latter were less than half the molarity of those of benzamide. In a typical experiment two digests were prepared, consisting of the buffer (pH 7·4) containing 0·21 g./100 ml. and 0·105 g./100 ml. benzamide respectively, together with the usual controls. The results of two experiments are shown in Fig. 3. Thus the percentage hydrolysis achieved is about 10 % greater with the more dilute substrate.

Effect of dialysis. The liver extract was prepared by the method described and its activity towards benzamide and *p*-nitrobenzamide determined. The remainder of the extract was dialyzed against running tap water for 17 hr. and its activity then determined, allowance being made for the dilution of the extract during dialysis.

The results of two experiments given in Fig. 4 show that there is no great difference in the equilibrium values reached. In almost all experiments in the main section undialyzed extracts were used.

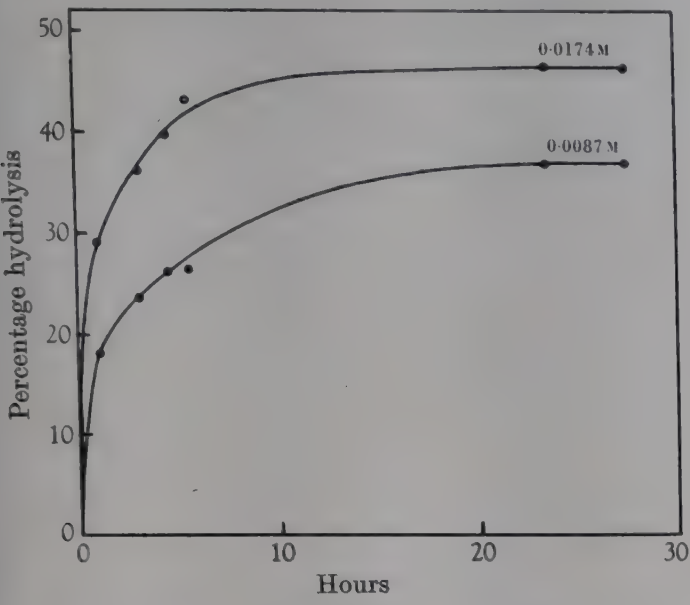

Fig. 3. Effect of concentration of substrate upon hydrolysis of benzamide by rabbit-liver extract.

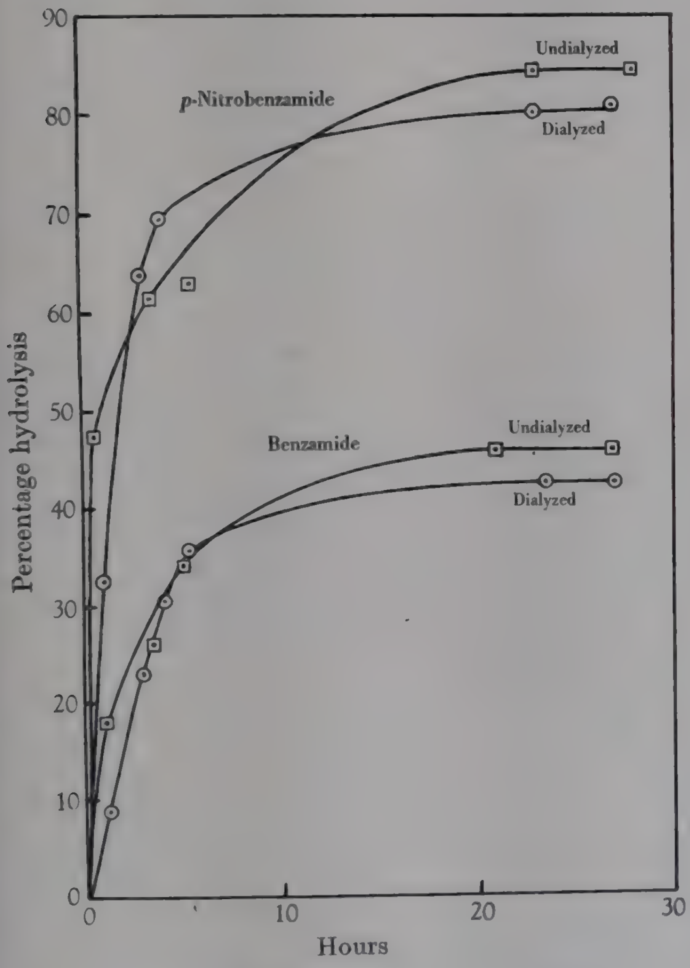

Fig. 4. Effect of dialysis upon activity of rabbit-liver extract.

Hydrolysis of various amides by liver extracts. The hydrolytic effect of liver extracts was studied using the three isomeric hydroxy-, amino- and nitrobenzamides, *o*-bromobenzamide, benzamide, formamide, acetamide, propionamide, phenylacetamide, nicotinamide, asparagine and glutamine. The results of typical experiments are shown in Fig. 5, and those of all experiments are summarized in Table 3.

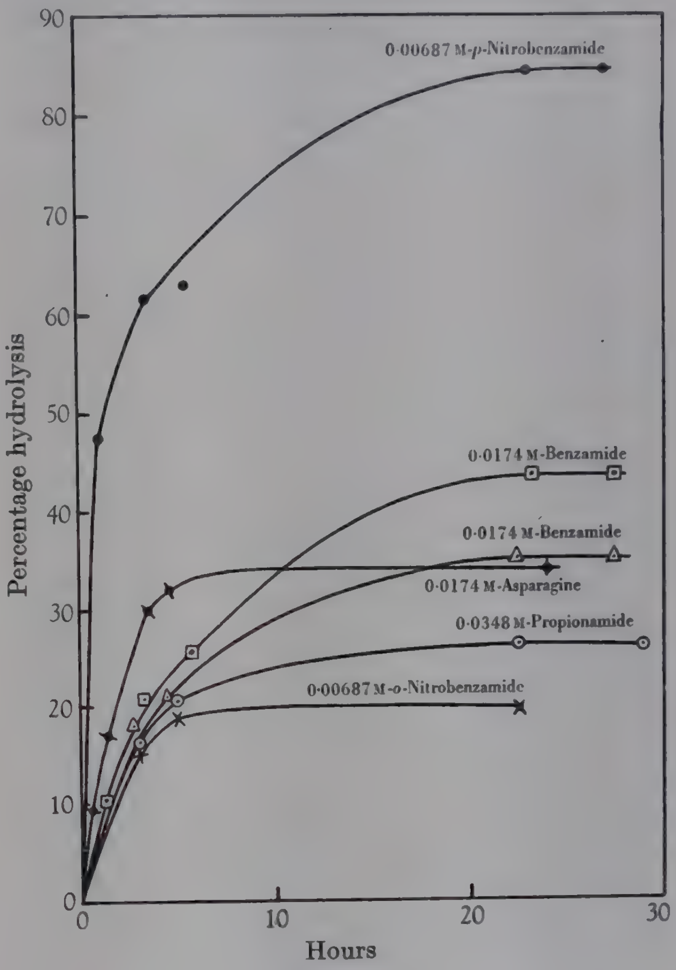

Fig. 5. Hydrolysis of amides by rabbit-liver extracts at pH 7·4.

Titration of ammonium salts. Solutions of the ammonium salts (25 ml., 0·0174 M) in buffer pH 7·4 were mixed with 5 ml. liver extract; 4 ml. samples of the mixture were treated with neutralized formaldehyde (0·5 ml. 40 %) and 0·1 N-NaOH (1 ml.) and centrifuged. Of the supernatant liquid 5 ml. were titrated against 0·02 N-NaOH. Blank determinations on the corresponding amides were carried out by adding liver extract (5 ml.) to 25 ml. of 0·0174 M-solutions of the amide in buffer pH 7·4; 4 ml. samples were withdrawn, treated with formaldehyde and titrated as above. The difference between the two titrations (corrected for volume) gives the titration due to the ammonia of the ammonium salt, and this represents the ammonia which would be formed by complete hydrolysis of the amide. The results shown in Table 4 justify the use of the theoretical value as the basis for the calculation of percentage hydrolysis. Incubation of the mixture of ammonium salt and liver extract at 37° for 18 hr. did not affect the titres.

Table 3. *Hydrolysis of amides by rabbit-liver extracts*

Amide	Hydrolysis		Average hydrolysis at equilibrium (%)
	In 10 hr. (%)	At equilibrium (%)	
Benzamide (average values)	24	45	45
o-Hydroxybenzamide	0	0	0
	0	0	
	0	0	
	0	0	
m-Hydroxybenzamide	0	0	1
	0	0	
	2	4	
p-Hydroxybenzamide	0	0	6
	0	0	
	3	10	
	7	13	
o-Aminobenzamide	0	0	9
	0	0	
	3	7	
	8	15	
	16	32	
m-Aminobenzamide	7	13	7
	0	4	
	0	5	
	0	0	
	3	13	
p-Aminobenzamide	2	8	13
	3	11	
	0	0	
	0	26	
	11	20	
o-Nitrobenzamide	12	27	23
	13	28	
	15	15	
m-Nitrobenzamide	12	36	31
	22	45	
	3	17	
	24	24	
p-Nitrobenzamide	93	100	82
	68	79	
	43	91	
	96	96	
	42	60	
	38	50	
	100	100	
	86	86	
	64	64	
	84	84	
	84	84	
	50	70	
	100	100	
o-Bromobenzamide	8	14	21
	0	28	
	5	22	
Formamide	0	0	0
	0	0	
Acetamide	3	6	2
	0	3	
	0	0	
Propionamide	24	32	25
	5	10	
	9	26	
	6	30	
Phenylacetamide	7	19	10
	6	7	
	0	0	
	8	15	
	4	8	
Nicotinamide	9	9	13
	8	8	
	8	26	
	9	9	
Asparagine	30	37	32
	29	29	
	30	32	
Glutamine	16	16	22
	28	28	

Table 4. *Formol titration of* 0·0174 M-*solutions of ammonium salts, expressed as ml.* 0·02 N-NaOH

	Titration	Difference	Difference corrected for volume*	Calculated difference
Ammonium benzoate	6·75	—	—	—
Benzamide blank	4·27	2·48	2·73	2·9
Ammonium *p*-nitrobenzoate†	5·5	—	—	—
p-Nitrobenzamide blank	4·4	1·1	1·21	1·14
Ammonium *p*-hydroxybenzoate	7·07	—	—	—
p-Hydroxybenzamide blank	4·75	2·32	2·55	2·9
Ammonium *p*-aminobenzoate	7·1	—	—	—
p-Aminobenzamide blank	4·2	2·88	3·17	2·9

* I.e. titration due to ammonium salt.

† 0·00687 M-solution used owing to small solubility of amide.

Experiments with kidney extract

The scope of these investigations was limited by the small amounts of tissue available. In general it may be said that kidney extracts possess feeble amide-hydrolyzing properties. The rate of hydrolysis in some cases was found to be very slow, e.g. in one experiment *p*-nitrobenzamide was hydrolyzed to an extent of 35 % in 30 hr. while, in another, 60 hr. was required to bring about the same change. It is, perhaps, significant that Waelsch & Busztin (1937) found that aqueous extracts of horse kidney showed no enzymic activity of this type, but that glycerol extracts were able both to hydrolyze and synthesize benzamide. We found that glycerol extracts of rabbit kidney were not more active than aqueous extracts.

Experiments with proteolytic enzymes of animal origin

Trypsin. Solutions of benzamide (0·21 g./100 ml.); *p*-hydroxybenzamide (0·23 g./100 ml.); phenylacetamide (0·23 g./100 ml.); and *p*-nitrobenzamide (0·115 g./100 ml.) in phosphate buffer pH 8·04 were prepared and to each 3 % pancreatin (British Drug Houses Ltd.) (10–50 ml.) was added. Similar solutions treated with boiled enzyme solution served as controls. A further control consisted of buffer and enzyme solution without substrate. The solutions were incubated at 37° and samples (4 ml.) withdrawn at intervals and titrated as before. In no case was any significant hydrolysis observed.

Pepsin. Solutions of benzamide, *p*-hydroxybenzamide, *p*-nitrobenzamide and phenylacetamide were prepared as in the last experiment, but using an acetate buffer pH 1·2. To 50 ml. of each solution was added 3 % pepsin (British Drug Houses Ltd.) solution (10 ml.). These were incubated along with controls as above and samples withdrawn and titrated as before. No significant hydrolysis was observed. Similar experiments at pH 5·9, using a phosphate buffer, also gave negative results.

Inhibition of enzyme action by various substances

The discrepancy observed between the complete hydrolysis of phenylacetamide which occurs *in vivo* (Bray *et al.* 1946) and its relative stability to liver extracts *in vitro* suggested that phenylacetamide or a metabolite, e.g. phenylacetic acid or phenaceturic acid, might have an inhibiting effect upon the enzyme responsible for amide hydrolysis. Benzamide and *p*-nitrobenzamide were used as substrates. It was found that 30 mg. of these substances/60 ml. digest produced significant inhibition. Typical results are given in Table 5.

Table 5. *Inhibiting effect of various compounds upon hydrolysis of benzamide and* p-*nitrobenzamide by liver extracts*

Amide	Compound added	Hydrolysis at equilibrium (%)
Benzamide	—	61
	Phenaceturic acid	43
	Phenylacetic acid	34
	Phenylacetamide	30
p-Nitrobenzamide	—	85
	Phenaceturic acid	75
	Phenylacetic acid	57
	Phenylacetamide	46

It may be noted that phenaceturic acid, the chief metabolite of phenylacetamide in the rabbit, shows less inhibiting effect than either phenylacetamide or phenylacetic acid.

Hydrolysis of amides by acid and alkali

In order to compare the stability of the amides to acid and alkali with their stability to liver extracts, a series of experiments was carried out in which they were incubated with acid and alkali under standard conditions and the amount of ammonia formed estimated by aeration into standard acid. The conditions chosen in the case of acid were heating 0·001 mol. with N-HCl (25 ml.) in a boiling-water bath for 1 hr. Under these conditions benzamide was hydrolyzed to the extent of 33 %.

The alkaline conditions were similar except for using N-NaOH for hydrolysis, and resulted in 73 % hydrolysis of benzamide. Table 6 shows the results, together with those obtained from the enzyme experiments. The order of stability to acid and alkali is not greatly different, but stability to enzymic hydrolysis clearly depends upon other factors.

Table 6. *Hydrolysis of amides by liver extracts, acid and alkali*

Amide	Hydrolysis by liver extract at equilibrium (%)	Hydrolysis by N-HCl in 1 hr. (%)	Hydrolysis by N-NaOH in 1 hr. (%)
p-Nitrobenzamide	82	35	79
Benzamide	45	33	73
Asparagine	32	50	—
m-Nitrobenzamide	31	27	81
Propionamide	25	87	78
o-Nitrobenzamide	23	1	21
Glutamine	22	—	—
o-Bromobenzamide	21	—	—
Nicotinamide	13	28	73
p-Aminobenzamide	13	30	54
Phenylacetamide	10	87	79
o-Aminobenzamide	9	8	64
m-Aminobenzamide	7	26	70
p-Hydroxybenzamide	6	19	14
Acetamide	3	88	75
m-Hydroxybenzamide	1	32	55
o-Hydroxybenzamide	0	9	9
Formamide	0	83	81

The mechanism of amide hydrolysis by enzymes

When acid and alkali were used for the hydrolysis of amides the ammonia formed was estimated, whereas in the enzyme experiments the formol titration gave a measure of the acid formed. If the mechanism of the enzymic reaction is, like that catalyzed by acid and alkali, a hydrolytic one expressed

$$R.\mathrm{CONH_2}+\mathrm{H_2O}\rightarrow R.\mathrm{COOH}+\mathrm{NH_3},$$

then both methods of estimation should yield the same result. To confirm this, the progress of hydrolysis in some enzymic digests was followed by both methods. Fig. 6 shows that the two methods yield essentially the same results. The fact that no increase in acidity of the enzymic digests was apparent until formaldehyde was added suggests that the mechanism is a hydrolytic one.

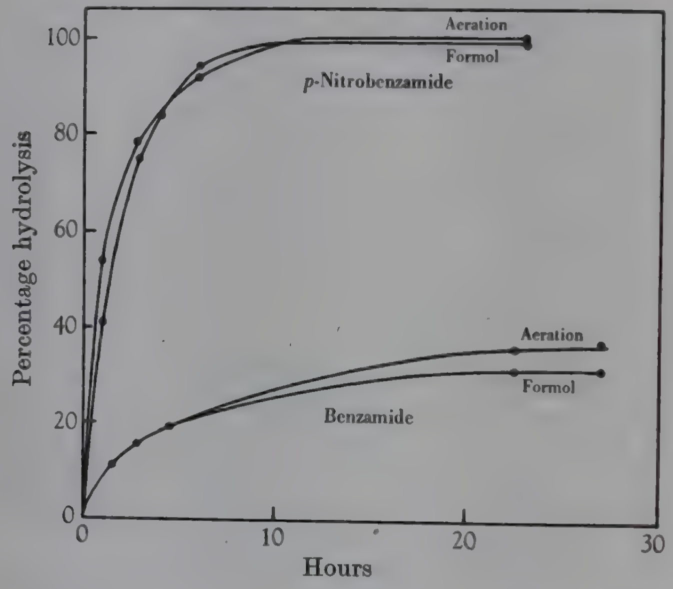

Fig. 6. Comparison of aeration and formol titration methods for estimation of hydrolysis of amides by rabbit liver extract.

The theoretical possibility of the enzymic conversion of the amide being due to an oxidative mechanism such as

$$R.\mathrm{CONH_2}+\mathrm{H_2O}+\mathrm{O}\rightarrow R.\mathrm{COOH}+\mathrm{NH_2OH},$$

with subsequent conversion of the hydroxylamine to ammonia, has, however, been explored to the extent of testing for the presence of hydroxylamine. The Pucher and Day reaction (Pucher & Day, 1926) applied to various digests gave negative results up to 48 hr. incubation. The amounts of hydroxylamine which could be formed, however, are near the lower limit of sensitivity of the test. Hydroxylamine is converted in the tissues into nitrite and ammonia (Lewin, 1888–9):

$$2\mathrm{NH_2OH}+\mathrm{O}\rightarrow \mathrm{HNO_2}+\mathrm{H_2O}+\mathrm{NH_3}.$$

The same change is brought about by liver extracts. Amounts of hydroxylamine sufficient to give a good Pucher and Day test were added to liver extracts (140 mg./50 ml.) and incubated for 20 hr. The extracts then failed to give a Pucher and Day reaction, but the presence of nitrite was easily detected by the Griess-Ilosvay diazotization test. This test applied to the various amide digests was, like the Pucher and Day test, always negative.

Further evidence against an oxidative mechanism, at least an aerobic one, was provided by comparing the results obtained with digests incubated under the stated conditions with those from digests incubated in an atmosphere of oxygen or nitrogen. No significantly different results were obtained.

Other properties of liver extracts

The liver extracts used for the experiments described in this paper were capable of reducing the three isomeric nitrobenzamides to yield diazotizable material. They were also able to oxidize hydroxybenzoic acids and amides to dihydroxy compounds (cf. Bray *et al.* 1947), which could be detected by means of the colorations given with ferric chloride. Both these properties of the extracts are being investigated in more detail.

Attempts to obtain active dried liver preparations. When dried by means of acetone and ether, rabbit liver yielded inactive preparations. Dried ox liver, however, showed some enzymic activity. These investigations are being continued.

DISCUSSION

The main object of this research was to compare the hydrolysis of amides *in vivo* with that by enzymes *in vitro*. The results set forth above show that in general the activity of liver extracts in hydrolyzing amides is similar to that of the intact animal, since we have already shown that the metabolism of benzamide is virtually indistinguishable from that of benzoic acid (Bray *et al.* 1946), and that *in vivo* the carbamyl group of *p*-hydroxybenzamide is hydrolyzed to only a very slight extent (Bray *et al.* 1947). Investigations in progress in this laboratory suggest that *in vivo* *o*- and *m*-hydroxybenzamides are incompletely, if at all, hydrolyzed, that the three isomeric aminobenzamides are hydrolyzed only partially (Bray, Lake, Neale, Thorpe & Wood, 1948), and that *m*- and *p*-nitrobenzamides are probably completely hydrolyzed. Nencki (1873) states that acetamide is excreted unchanged by dogs, although his evidence does not preclude the possibility of a slight degree of hydrolysis.

So far, phenylacetamide appears to be the only exception to this general agreement, inasmuch as it is completely hydrolyzed *in vivo** (Bray *et al.* 1946), but only to an extent of 10 % by liver extracts. This apparent discrepancy may not be as great as it appears at first, since under the dynamic conditions prevailing in the living animal products of a reaction of this type are continuously removed, so that it can proceed further towards completion than under static conditions such as those used in *in vitro* experiments. Thus it is possible that amides which appear to be hydrolyzed with difficulty *in vitro* could be completely hydrolyzed *in vivo*. Phenylacetamide is excreted (as phenaceturic acid) much more slowly than benzamide or benzoic acid (as hippuric acid). Since, however, phenylacetic acid is eliminated as slowly as phenylacetamide, it would seem that it is the conjugation with glycine which is delayed rather than the hydrolysis of the amide.

The fact that the liver extracts used in this study were active in hydrolyzing asparagine and glutamine suggests that it might be profitable to investigate the possible identity of the liver enzyme(s) with asparaginase or glutaminase.

SUMMARY

1. The hydrolysis of 18 amides by rabbit-liver extracts has been studied and the results compared with some obtained on the rabbit *in vivo*.

2. A suitable pH for the reaction was 7·4.

3. Rabbit-kidney extracts behave in a qualitatively similar manner, but are less active.

4. The action of papain, pepsin and trypsin upon some of the amides is recorded.

5. There is considerable difference between the stability of the amides to hydrolysis by HCl or NaOH and by liver extracts.

We are indebted to the Royal Society for a Government Grant which defrayed part of the cost of this work. The results for the percentage hydrolysis of amides by acid and alkali were obtained by Mr P. B. Wood, whom we wish to thank.

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* These findings have been confirmed during the present investigation.

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Leaf Protease of Tobacco and other Plants

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The work described in this paper was undertaken to obtain information on the properties and activities of protein- and peptide-splitting enzymes of the green leaves of tobacco and other plants. These enzymes will be referred to as proteases.

Greenberg & Winnick (1945) list eleven plant proteases that have been obtained in a relatively high state of purity. All, with the exception of bromelin, pinguinain and solanain, are obtained from latex. These three are obtained from fruits, although bromelin also occurs in the green leaves of the pineapple. Other plant proteases which have been studied in detail are those of seeds, both dormant and germinating. Reports on proteases in the green leaves of plants that do not produce latex are few, and frequently amount to little more than an assertion of their presence, based in much of the early work on evidence that seems open to question.

The presence of proteases in the green leaf of tobacco has been noted by Loew (1900) (action on egg albumin; none detected on casein, fibrin or gelatin); Oosthuizen & Shedd (1913) (possibly present in cured leaves); Fodor & Reifenberg (1925, 1926) (present in green and dried leaves, peptone substrate); Silberschmidt (1934) (stem sap of *Nicotiana-Datura* grafts, peptone substrate); and by Nito & Kitamura (1936) ('trypsin' and 'erepsin' in fresh leaves, 'trypsin' lost on drying). Much of the difficulty in working on the protease of green leaves is due to its low concentration—the activity/g. protein N of tobacco sap is seldom as high as 1% of that of a commercial papain preparation.

MATERIALS AND METHODS

Leaves of *Nicotiana tabacum* var. White Burley from glasshouse plants were used for most of the work, but leaves of other plants listed later were also used. The leaves were minced twice in a domestic meat mincer and the sap squeezed by hand through madapollam. The crude sap was freed from much cell debris and chloroplast material by centrifugation for 15 min. at 3000 r.p.m. (*c.* 1500 *g*); the supernatant fluid is subsequently referred to as 'sap'. The fibrous residue remaining after the expression of the crude sap was washed twice with a volume of distilled water approximately equal to that of the crude sap, and squeezed dry by hand. The residue is referred to as 'fibre'.

Measurement of protease activity

Changes in the viscosity of gelatin due to the action of protease were first used, but were abandoned as the low activity necessitated long periods of measurement during which spontaneous precipitation occurred leading to inaccuracy. The method finally adopted was that of measuring the increase of amino N produced by the incubation at 40° of protease-containing material with 0·8% (w/v) gelatin at pH 5. Michaelis buffer was used because it has a wide useful range and, unlike citrate, does not interfere with the subsequent estimation of amino N. Amino-acid N was determined by a modification of the method of Pope & Stevens (1939) which allows the use of small quantities of test material and eliminates interference by reducing materials.

Specificity of the method of Pope & Stevens (1939)

The method depends on the decomposition of copper phosphate at a controlled pH by amino-acids with the formation of amino-acid-copper complexes. Peptides also behave in the same way and their N is included in the amino N figures obtained. Since the method is here used for the estimation of extent of protein splitting, this lack of specificity is immaterial. Pope & Stevens found that about 80% of the amino N of completely hydrolyzed gelatin was estimated by this method. They used solutions containing from 0·028 to 0·4 mg. amino N/ml. after final dilution with buffered copper phosphate, and found that low results were obtained in the lower part of this range. In this work, final concentrations were only of the order of 0·004–0·024 mg. amino N/ml. and it was found that in this range about 80% of amino N was estimated when added as glycine which is all estimated at higher concentrations. Nevertheless the method is useful at these low concentrations and gives reproducible results.

A number of substances present in plant sap and fibre may be estimated as amino N, but would not interfere in the measurement of protease activity if their concentration remained unchanged during incubation, e.g. sugars, pectin and pectic acid, and organic acids. These are considered below.

Sugars. Sucrose and reducing sugars may account for up to 10% of the leaf dry weight and non-cellulosic polysaccharides (mainly starch) for a further 20–30% (Ward, 1942). Glucose and sucrose were found not to interfere in the concentrations likely to be encountered; further confirmation was obtained by adding salivary amylase to sap containing starch and soluble sugars, and incubating in the usual way with gelatin. At the end of incubation the amylase was still active in degrading added starch, but had produced no apparent increase in the protease activity measured.

Pectic acid. Methanol is present in tobacco sap (Holden, 1945) and its concentration (0·05 mg./ml.) indicates the possibility of the presence of about 0·5 mg./ml. of pectic acid. It was thought that the many free carboxyl groups of pectic acid might interfere. Its formation during incubation would be likely to interfere only with sap protease estimations, since when formed in fibre it appears to remain attached (Holden, 1945). It was found that pectic acid at a conc. of 1 mg./ml. did not increase the blank values.

Organic acids. Of the organic acids present in the leaf that are likely to interfere, oxalic may be excluded since it is not soluble at the pH values used (Vickery & Pucher, 1931). Malic acid does not interfere at the concentrations likely to be encountered, but citric acid does. Since malic acid may be converted to citric acid in water-cultured leaves in the dark (Vickery, Pucher, Wakeman & Leavenworth, 1937) interference from this cause must be considered. Most of the protease estimations were carried out in the presence of a reducing agent which lessens the likelihood of the change from malic to citric acid. It was found that the addition of 1 mg. of malic acid/ml. of sap did not affect the protease value found, and that sap dialyzed against 0·02 M-thioglycollic acid at 5° had lost no activity in spite of the loss of 75% of its initial 'amino N' content, and hence presumably of a major portion of its organic acids.

Amino-acid oxidase. If this were present low results would be obtained owing to destruction of amino-acids. Estimations of ammonia were made on samples incubated in the usual way and for twice the usual time and the difference between initial and final values was no more than 0·01 mg./ml.

Method of estimation

The enzyme solution (0·5 ml.) was pipetted into a 15 ml. tube and 0·4 ml. of distilled water added, followed by 0·1 ml. of a M-solution of activator or inhibitor. A 1% (w/v) gelatin solution (4 ml.) in Michaelis buffer (pH 5·0) at about 30° was then added and the contents of the tube mixed. A 2 ml. sample was then withdrawn into a small beaker, two drops of starch indicator added followed by iodine until the development of a permanent blue colour. The iodine stops the action of the enzyme and removes any reducing substances that would interfere in the subsequent titrations. After 30 min. excess iodine was removed quantitatively with 0·005 N-thiosulphate. From this point the procedure of Pope & Stevens (1939) was followed except that 15 ml. of copper phosphate suspension were used and the samples brought to a final volume of 25 ml. before filtration. Titration was carried out using 0·005 N-thiosulphate and a 1 ml. burette delivering below the surface of the fluid; 5 ml. samples of the filtrate were used with the addition of 0·25 ml. of glacial acetic acid and 0·5 ml. of saturated KI. With starch indicator, the end point was not very sharp and increased accuracy could be obtained by the electrometric titration of Foulk & Bawden (1926) as modified by Bonner (1946). Gelatin and water blanks were run with each set of estimations, and for absolute values determinations were made on glycine solutions.

Immediately after the initial samples had been taken a drop of chloroform was added to each tube, the tubes stoppered and incubated at 40° for 24 hr. At the end of this time, a second 2 ml. sample was taken and treated in the same manner as the first. From the difference in the values obtained by titration of initial and final samples the number of units/ml. was calculated. If the activity of fibre is to be estimated 1 g. is weighed into the tube and double amounts of water, activators or inhibitors and gelatin solution are added. Samples of 2 ml. are taken and treated like the samples from estimations in liquids.

The unit of activity is defined as that activity which leads to the production of 1 mg. of amino N, as estimated by the method used, on incubation at pH 5 for 24 hr. at 40°. The protease unit is the amount of protease which produces 1 mg. of amino N, as estimated by the method used, on incubation at pH 5 with 0·8 % (w/v) gelatin for 24 hr. at 40°.

RESULTS

Preliminary observations on the protease

Protease activity is shown both by sap and fibre of tobacco and other leaves, about 60 % of the total activity of healthy tobacco leaves being present in the sap. The activity of both sap and fibre was increased by reducing agents such as sodium thioglycollate, the reagent most frequently used in routine experiments. Activators and inhibitors were used at a final concentration of 0·02 M. Sodium sulphide, sodium hyposulphite, potassium cyanide, ascorbic acid, cysteine and glutathione were also effective as activators. Activity was unaffected by manganese, was reduced to about 25 % of its thioglycollate value by iodoacetic acid and was abolished by copper. The pH optimum of the protease was found to be *c.* 5 with gelatin as substrate. The enzyme has thus many properties in common with other plant enzymes of the papain type.

Method of partial purification

If tobacco sap is heated to 55° for a few minutes much of the normal plant protein is denatured and can be removed by centrifugation (Bawden & Pirie, 1938). Such heating leads to some loss of total activity, but may increase the purity (i.e. activity/g. protein N) by as much as tenfold. The addition of one twentieth volume of ethanol to heated sap adjusted to pH 4·2 gives a further increase in purity. Table 1 shows the progress of a typical purification. The purification at each step may vary widely, but the overall results are usually similar to the example given. Precipitation of the enzyme has been attempted under many conditions, but does not seem to give as satisfactory results as methods based on the precipitation of impurities. Attempts have been made to prepare fractions of sap protein with enhanced protease content by using $(NH_4)_2SO_4$, ethanol and acetone. These have not been successful, all the fractions prepared having much the same activity. Protease present on fibre is not removed by water, 5 % NaCl or glycerol solutions and has not been purified.

Properties of the enzyme

Fig. 1 shows the variation of the activity of the protease with the pH of the medium. Determinations were made by adding buffered 1 % gelatin to samples of sap, adding activator and water and determining the pH after the initial and final samples were taken. Michaelis buffer was used.

Variation of activity with substrate concentration was measured in the usual way. Difficulty was encountered in handling samples containing more

Table 1. *Partial purification of tobacco-sap protease*

	Protease (units/ml.)	Protein N (mg./ml.)	Protease (units/g. protein N)	Total protease (units)	Protease units as % of initial value
(*A*) Crude sap	0·39	2·22	176	35·1	100
(*B*) *A* spun*	0·40	1·82	247	34·8	99
(*C*) *B* heated and spun*	0·28	0·82	342	21·8	62
(*D*) *C* spun at pH 4·2*	0·25	0·34	735	18·2	52
(*E*) *D*+5% (w/v) ethanol, spun*	0·21	0·23	910	15·3	44
(*F*) *E* concentrated by ultrafiltration and dialyzed	—	—	—	—	—
(*G*) Fraction of *F* insoluble in Michaelis buffer, pH 5	0·09	0·175	515	2·1	6
(*H*) Fraction of *F* soluble in Michaelis buffer, pH 5	0·33	0·11	3000	7·65	22

* Refers only to the supernatant liquid.

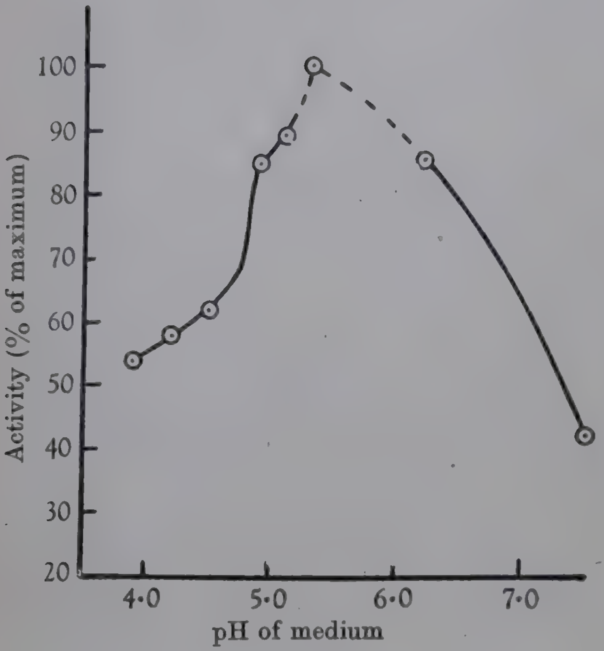

Fig. 1.

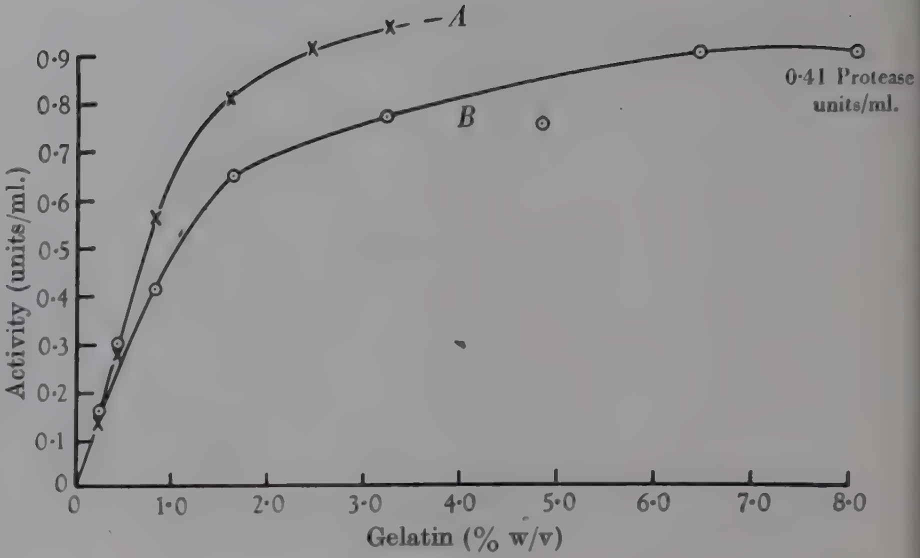

Fig. 2.

Fig. 1. Protease activity at various pH values expressed as percentage of the maximum activity found. Portions of the same sample of partially purified tobacco sap were used for all determinations.

Fig. 2. Variation of activity with substrate concentration in partially purified samples of tobacco-sap protease containing (*A*) 0·56 and (*B*) 0·41 protease units/ml.

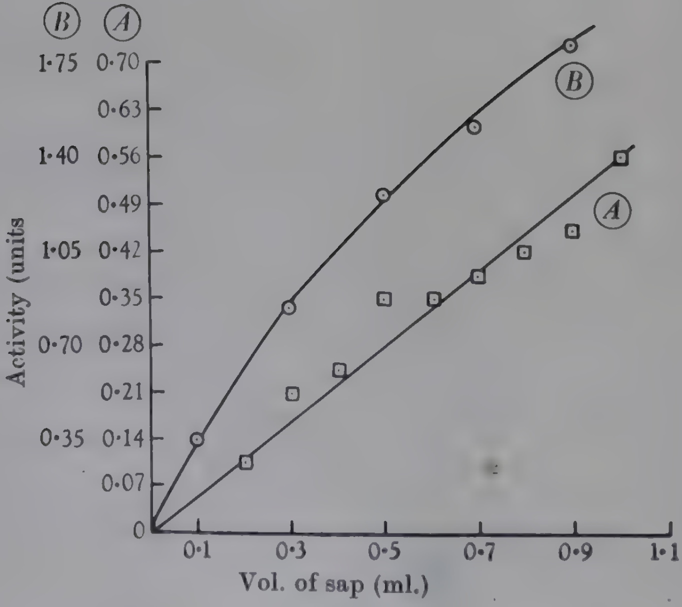

Fig. 3.

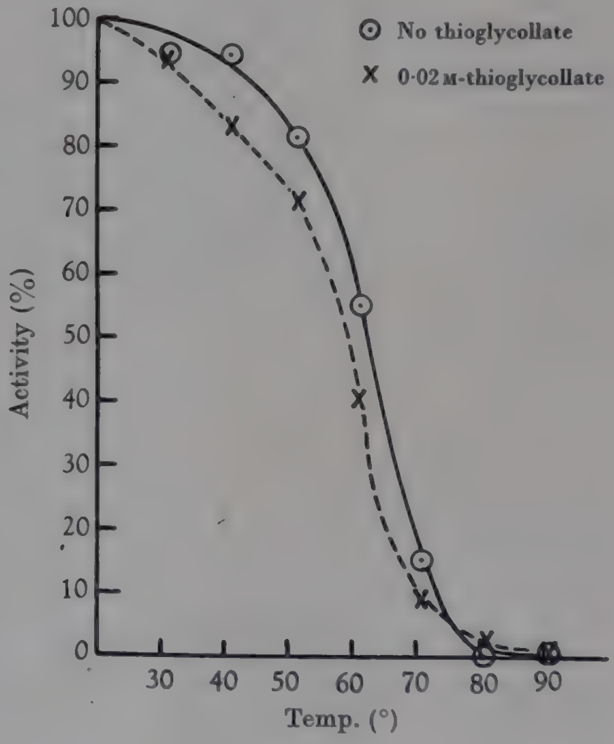

Fig. 4.

Fig. 3. Variation of activity with protease concentration, (*A*) 0·28 protease unit/ml., (*B*) 1·22 protease units/ml.

Fig. 4. The effect of heat on tobacco-sap protease. Portions of partially purified protease were heated for 5 min. at the temperatures indicated, in the absence of added substrate. Activities subsequently measured are expressed as percentages of that of the unheated sap.

than 4% (w/v) gelatin owing to gelling during sampling. Typical results are shown in Fig. 2. The concentration of gelatin which gives half the maximum velocity of hydrolysis is about 0·8%.

An approximately linear relationship was found between the amount of enzyme present and the extent of hydrolysis over the enzyme concentration range usually employed (Fig. 3). The effect of heat on enzyme activity was determined by heating samples of sap at pH 5 in the absence of added substrate to a desired temperature, maintaining it at that temperature for 5 min. and cooling rapidly. The activity of the heated samples was then measured in the usual manner. Fig. 4 shows that some inactivation occurs even at 40° and that the presence of reducing agent has little effect. The influence of the temperature of incubation on the activity measured was measured at four temperatures. Enzyme action at 25° was 57%; at 20°, 25%; at 5°, 14% of the activity at 40° using gelatin as substrate. The pH of maximum stability on storage at 5° was measured on samples of tomato sap. Values of pH were measured 1 hr. after adjustment and at the end of 20 hr. at 5° and were found not to have changed, except for the sample initially at 6·5 which had changed to 6·3 and the sample at 7·4 which had changed to 7·0. Final pH values have been used in Fig. 5. The pH of maximum stability seems to be in the region of the optimum for protease activity, about pH 5.

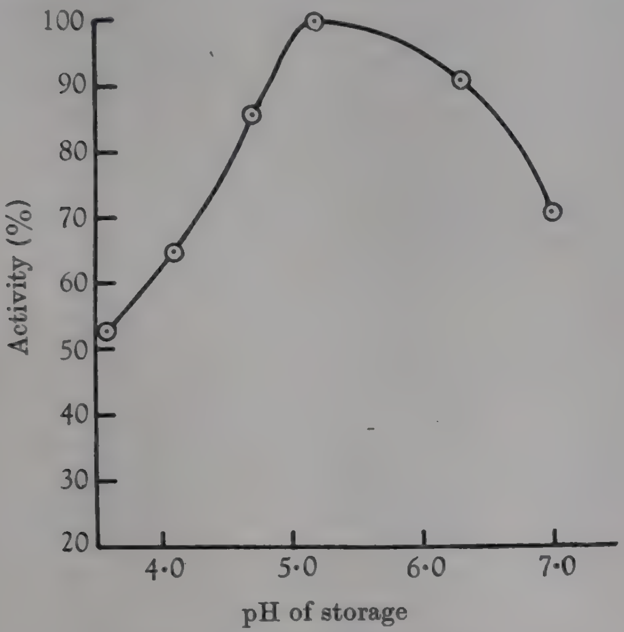

Fig. 5. The effect of pH on the stability of tomato-sap protease kept at 5° for 20 hr. The activities are expressed as percentages of the maximum observed activity in this experiment.

The course of protease activity in the presence of added substrate was followed for both sap and fibre for 7 days at 40°. In Fig. 6 the values for the sap have been multiplied by 3·3 in order to show an equivalent activity.

A number of substrates have been tested with tobacco-leaf protease. The protein present in both crude and spun sap is split to some extent, but the amount of amino N released is small compared with that which occurs in the presence of added gelatin.

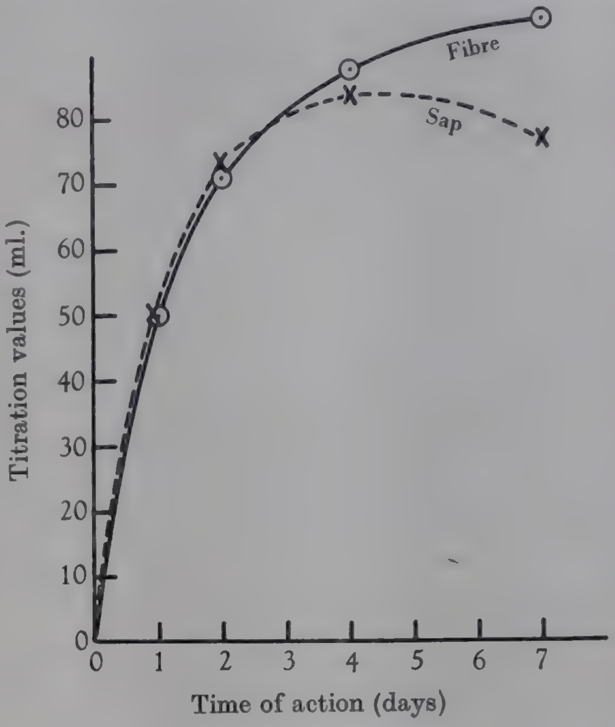

Fig. 6. Progress curve for the hydrolysis of gelatin (0·8%, w/v) by tobacco protease over an extended period. Titration values for equal samples withdrawn at intervals are plotted against time. The values for sap have been multiplied by 10/3.

Table 2. *Activity of tobacco protease with various substrates*

	(units/ml.)
Crude sap*	0·18
Spun sap†	0·03
Spun sap + gelatin	0·45
Spun sap + wheat gluten	0·40
Spun sap + egg albumin	0·07
Spun sap + peptone	0·45
	(units/g. dry wt.)
Washed fibre	3·10
Washed fibre + gelatin	4·50

* Final concentration of sap protein *c.* 0·14%.
† Final concentration of sap protein *c.* 0·11%.

Table 2 shows the amount of amino N formed by tobacco protease acting on various substrates and its own proteins at pH 5. Added substrates had a final concentration of 0·8% (w/v); with sap alone the usual volume of 0·5 ml. was used.

Inhibition by iodoacetate

Tobacco-leaf protease is not completely inhibited in the presence of 0·02 M or even 0·1 M-iodoacetate though no activity can be detected in the presence of 0·02 M-copper salts. It is possible that the protease is a mixture of enzymes, some stable to iodoacetate and some inhibited by it. The ratio of full activity in the presence of thioglycollate to the residual activity shown in the presence of iodoacetate does not change appreciably during the course of preliminary purification, and attempts to demonstrate the existence of different pH optima in the presence of thioglycollate and the presence of iodoacetate have not been successful.

Leaf protease of other plants

The leaves of a number of plants have been examined for the presence of protease. In all those so far examined, the enzyme is activated by thioglycollate and is active at pH 5. Table 3 lists some of the plants tested. In those for which a range of values is given, samples varying in fertilizer treatment were used. The leaves of some plants do not yield sap on mincing (hollyhock, *Althea rosea*; box, *Buxus sempervirens*), while laurel (*Prunus laurocerasus*) gives a sap containing only traces of protein. These leaves were however found to contain protease in amounts similar to those in tobacco.

Table 3. *Sap and fibre protease content of a number of plants*

		Sap (units/ml.)	Sap (units/g. protein N)	Fibre (units/g. dry wt.)	Fibre (units/g. N)
Solanaceae:					
Tobacco, *Nicotiana tabacum*	(1)	0·03–0·63	25–1040	0·8–12·4	20–270
Potato, *Solanum tuberosum*	(2)	0·60–2·18	150–780	8·6–13·8	160–390
Woody nightshade, *Solanum dulcamara*		1·02	1020	—	—
Tomato, *Lycopersicum esculentum*		0·79	370	—	—
Chenopodiaceae:					
Sugar beet, *Beta vulgaris*	(3)	0·14–0·67	60–400	1·8–6·7	70–320
Cucurbitaceae:					
Marrow, *Cucurbita pepo* var. *ovifera*		0·42	244	1·2	51
Bryony, *Bryonia dioica*		0·21	130	1·6	31
Cruciferae:					
Cabbage, *Brassica oleracea*		0·35	263	12·5	330
Leguminosae:					
Bean, *Phaseolus vulgaris*		0·63	375	1·02	133
Gramineae:					
Wheat, *Triticum vulgare*	(4)	2·7–4·1	350–580	2·5–3·5	120–210
Cocksfoot, *Dactylis glomerata*		0·14	202	0·63	77
Bromeliaceae:					
Pineapple, green leaves of *Ananas sativus*		6·5	13,000	8·85	—
Papain (British Drug Houses, 0·005 mg. N/ml.)		0·42	84,000	—	—

Results are from single determinations except (1)–(4) which are the ranges with (1) 80 samples, (2) 16 samples, (3) 54 samples, (4) 6 samples of plants with varied fertilizer treatments at different seasons.

DISCUSSION

The methods described in this paper have been used to measure the protease content of plants grown under different fertilizer conditions and infected with different viruses. It is hoped to publish these results shortly. They show that very wide variations in protease content may be found in plants grown under different cultural conditions (Table 3: tobacco, potato, beet), and that caution must therefore be exercised in drawing conclusions from the examination of plants of unknown antecedents and cultural background. In spite of these variations in protease concentration, none of the plants examined has approached the protease content of the pineapple, which is typical of those plants containing the well characterized plant proteases. The plants examined do, however, appear to form a homogeneous group when protease contents are compared on a basis of units/g. protein N, or units/g. total N. All the values quoted for sap protease/g. protein N (with the exception of pineapple) fall within the range found in tobacco, although the concentrations in sap on a volume basis may differ by a factor of six.

It is probable that the proteases described in this paper have not been examined in any detail before because they occur in such small concentrations. It must be assumed, however, that the protease levels found are adequate for the metabolic needs of the plants; if the total plant protein were equivalent as substrate to gelatin, all the leaf protein could be split by its enzymes in 2–10 days at pH 5 and 40°. Thus it appears that the levels in some plants are abnormally high. It is difficult to see what evolutionary advantage the remarkably high protease activity in latex and fruits has for the plants concerned, since an abnormal resistance to pathogens does not seem to be conferred; pineapple, papaw and fig all suffer from virus diseases.

No evidence has been found to show that the rotease is a mixture of enzymes or that the protease f one plant is distinguishable from another. It has een customary to name a protease after the generic ame of the plant in which it is found. This custom, oth here and in future work, implies an indefinite xtension in the number of proteases. It is therefore ecommended that the proteases described in this paper shall be considered identical until evidence to the contrary is adduced, and that they be called leaf proteases, adding where necessary the name of the plant.

SUMMARY

1. Protease activity is shown by both sap and fibre of the green leaves of the plants examined.

2. This activity is less than one tenth of that shown by the leaves of pineapple.

3. The activation of the enzyme by reducing agents and the pH optimum suggest that the protease is similar to the papain enzymes.

4. It is suggested that the enzyme be referred to as 'leaf protease' until evidence is obtained of the existence of more than one protease in the leaf or of differences between the leaf proteases of different plants.

5. The method of Pope & Stevens (1939) has been modified to allow smaller amounts of amino nitrogen to be estimated in the presence of reducing agents.

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The Free Amino Groups of Haemoglobins

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Since the original work of Reichert & Brown (1909) on the crystal form of the haemoglobins from a large number of species, many workers have shown them to differ in chemical and physical properties. Among the properties studied were the CO-combining capacity (Douglas, Haldane & Haldane, 1912), oxygen dissociation (Macela & Seliškar, 1925), absorption spectra (Anson, Barcroft, Mirsky & Oinuma, 1925), amino-acid composition (Vickery & White, 1933; Block, 1934), rate of alkali denaturation (von Krüger, 1887) and electrophoretic mobility (Landsteiner, Longsworth & van der Scheer, 1938). Apart from the occasional ability of haemoglobins of closely related species such as the horse and the donkey to form mixed crystals, the property which has brought out marked similarities as well as differences is their immunological behaviour. The most extensive survey was made by Hetkoen & Boor (1931), who showed that, although the haemoglobins were species-specific, cross reactions occurred between the antibodies and antigens of allied species. The extensive work of Landsteiner (1945) and others has suggested a relationship between the antigenic behaviour of a protein and its chemical structure. As an investigation of the free α-amino-groups of a protein brings out some details of the molecular structure (Sanger, 1945), it was of interest to make a comparative study of the terminal residues of several haemoglobins, and to see if any correlation between structure and immunological behaviour could be detected.

EXPERIMENTAL

Preparation of DNP-amino-acids

The method for the preparation of 2:4-dinitrophenylamino-acids (DNP-amino-acids) using 1:2:4-fluorodinitrobenzene (FDNB) has been previously described (Sanger, 1945).

Details for the purification of the various derivatives are given below.

N-2:4-*Dinitrophenyl*-DL-*isoleucine*. The amorphous product obtained by acidifying the alkaline reaction mixture was filtered off and crystallized from methanol-water; m.p. 166°. (Found: C, 48·1; H, 4·9%. $C_{12}H_{15}O_6N_3$ requires C, 48·6; H, 5·0%.)

N-2:4-*Dinitrophenyl*-DL-*methionine*. On acidification of the reaction mixture an oil separated which was extracted into ether. The ether extract was dried over anhydrous Na_2SO_4 and taken to dryness leaving an oil which crystallized overnight. Yield 80% theoretical. The material was recrystallized by dissolving in dry ether and adding an equal volume of light petroleum (b.p. 80–100°). On evaporation of the ether crystalline plates were obtained, m.p. 117°. (Found: C, 41·9; H, 4·2; N, 13·3%. $C_{11}H_{13}O_6N_3S$ requires C, 41·9; H, 4·1; N, 13·3%.)

N-2:4-*Dinitrophenyl*-L-*proline*. On acidification an oil was formed which solidified overnight. Yield 85% theoretical. After recrystallization from ether-light petroleum and acetic acid-water it had m.p. 137°. (Found: C, 46·8; H, 3·9; N, 14·6%. $C_{11}H_{11}O_6N_3$ requires C, 47·0; H, 3·9; N, 14·9%.)

N-2:4-*Dinitrophenyl*-L-*tryptophan*. A gum which was obtained on acidification crystallized readily from aqueous methanol; m.p. 175° (decomp.). (Found: C, 55·5; H, 3·7%. $C_{17}H_{15}O_6N_4$ requires C, 55·0; H, 3·8%.)

NN′-*bis*(2:4-*Dinitrophenyl*)-L-*lysine*. The amorphous precipitate obtained on acidification was crystallized from formic acid with some difficulty and was recrystallized from methanol-water; m.p. 146°. (Found: C, 45·2; H, 3·73; N, 17·8%. $C_{18}H_{18}O_{10}N_6$ requires C, 45·1; H, 3·77; N, 17·6%.)

N-2:4-*Dinitrophenyl*-DL-*aspartic acid*. This compound crystallized on acidification and was recrystallized from water; m.p. 196° (decomp.). (Found: C, 40·2; H, 3·0; N, 13·9%. $C_{10}H_9O_8N_3$ requires C, 40·2; H, 3·0; N, 14·0%.)

N-2:4-*Dinitrophenyl*-DL-*serine*. The oil which separated on acidification crystallized on standing and was recrystallized from methanol; m.p. 199°. (Found: C, 39·5; H, 3·2; N, 15·4%. $C_9H_9O_7N_3$ requires C, 39·8; H, 3·3; N, 15·5%.)

N-2:4-*Dinitrophenyl*-DL-*threonine*. Crystallized on acidification and recrystallized from aqueous methanol; m.p. 152°. (Found: C, 41·7; H, 3·8%. $C_{10}H_{11}O_7N_3$ requires C, 42·1; H, 3·8%.)

NN′-*bis*(2:4-*Dinitrophenyl*)-L-*cystine*. On acidification an amorphous precipitate separated. It was crystallized from ethylene glycol monoethyl ether-water and recrystallized from acetic acid-water. Yield 80% theoretical; m.p. 109°. (Found: C, 38·2; H, 3·1; S, 11·2%. $C_{18}H_{16}O_{12}N_6S_2$ requires C, 37·8; H, 2·8; S, 11·2%.)

N^1-2:4-*Dinitrophenyl*-L-*arginine*. After removal of the ethanol from the reaction mixture, the residue was treated with water. *DNP-arginine* was insoluble and was filtered off and washed with ethanol and ether. It was recrystallized by solution in dilute HCl and neutralization with ammonia; yield 70%, m.p. 252° (decomp.). For analysis it was crystallized from conc. HCl as the hydrochloride; m.p. 168°. (Found: C, 37·7; H, 4·4; N, 22·5%. $C_{12}H_{16}O_6N_6.HCl$ requires C, 38·3; H, 4·5; N, 22·6%.)

The DNP-derivatives of glutamic acid and hydroxyproline have not yet been obtained in crystalline form.

N^5-2:4-*Dinitrophenyl*-L-*lysine*. A simpler preparation of this compound similar to that described for DNP-ornithine (Sanger, 1946) has now been achieved. L-Lysine (0·5 g.) was dissolved in 10 ml. water and $CuCO_3$ added slowly to the boiling solution. The excess $CuCO_3$ was filtered from the dark blue solution and washed with 2–3 ml. water. Excess $NaHCO_3$ was added followed by a solution of 1·5 g. DNFB in 20 ml. ethanol and the mixture shaken for 2 hr. at room temperature. The yellow-green precipitate was filtered off and washed with water, ethanol and ether. It was then suspended in 5 ml. water and sufficient N-HCl added to produce a clear solution. This solution was cooled in ice, H_2S bubbled through for 2 min., a trace of charcoal added and the mixture filtered immediately. The filtrate was rapidly taken to dryness *in vacuo*, crystallized from water and recrystallized from 20% HCl; yield 0·45 g.

Chromatographic separation of DNP-derivatives

The methods of separation and identification of the DNP-derivatives are essentially the same as those previously described (Sanger, 1945). It should be emphasized again that the R_F values recorded in that paper apply only to a certain batch of silica gel (cf. Tristram, 1946). The absolute, and to a lesser extent the relative, R_F values vary considerably with different batches of gel. It appears that the DNP-amino-acids are partly held in the aqueous phase by adsorption on the silica and that the fractionation is not entirely the result of liquid-liquid partition. In general an adsorbent gel, giving low R_F values, produces the maximum resolution for any given solvent, though increased 'tailing' of the bands sometimes prevents clean separation. Table 1 shows a scheme of fractionation which has been found applicable to most batches of gel used in this laboratory.

Stability of DNP-amino-acids to acid hydrolysis

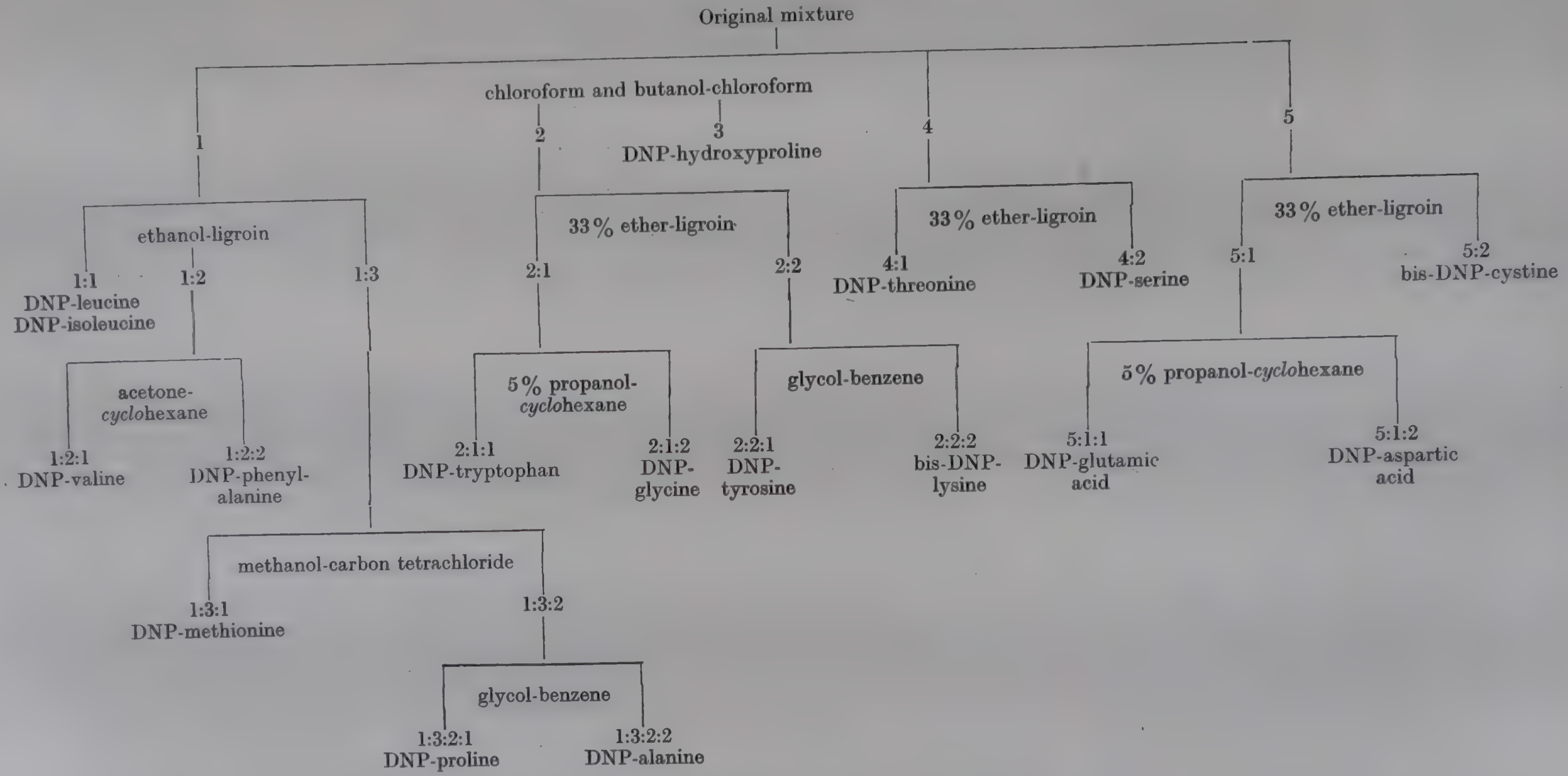
Hydrolysis in boiling 5·7 N-HCl. In order to ascertain whether any terminal amino-acids might be overlooked due to the instability of their DNP-derivatives during hydrolysis, a survey was made of the rates of breakdown of all the DNP-derivatives.

A standard solution was made of DNP-amino-acid in 5·7 N-HCl. A suitable portion was taken, some globin was added in case the rate of breakdown was influenced by the presence of hydrolytic products of protein, and the mixture boiled under reflux for the required time. The hydrolysate was extracted as previously described and the extract passed through appropriate columns in order to separate any breakdown products. The DNP-amino-acid was collected and the breakdown estimated by colorimetric comparison with the original solution. The results obtained are shown in Table 2.

It is clear that, with the exception of proline (Consden, Gordon, Martin & Synge, 1946), all amino-acids with a free amino or imino group could be detected using this method of hydrolysis. It was found, however, that during hydrolysis, some DNP-amino-acids gave rise to coloured breakdown products which were liable to cause confusion in the chromatographic separation. DNP-methionine and DNP-tyrosine both produced a coloured 'artefact' band stationary on a chloroform column. DNP-cystine yields a decomposition product which hardly moves on a 3% butanol-chloroform column. DNP-tryptophan on hydrolysis gave

Table 1. *Scheme of fractionation of ether-soluble DNP-amino-acids*

(Numbering refers to fractions and subfractions.)


Original mixture
chloroform and butanol-chloroform
1
2
3
DNP-hydroxyproline
4
5
ethanol-ligroin
1:1
DNP-leucine
DNP-isoleucine
1:2
1:3
acetone-
*cyclo*hexane
1:2:1
DNP-valine
1:2:2
DNP-phenyl-
alanine
methanol-carbon tetrachloride
1:3:1
DNP-methionine
1:3:2
glycol-benzene
1:3:2:1
DNP-proline
1:3:2:2
DNP-alanine
33 % ether-ligroin
2:1
2:2
5 % propanol-
*cyclo*hexane
2:1:1
DNP-tryptophan
2:1:2
DNP-
glycine
glycol-benzene
2:2:1
DNP-
tyrosine
2:2:2
bis-DNP-
lysine
33 % ether-ligroin
4:1
DNP-threonine
4:2
DNP-serine
33 % ether-ligroin
5:1
5:2
bis-DNP-cystine
5 % propanol-*cyclo*hexane
5:1:1
DNP-glutamic
acid
5:1:2
DNP-aspartic
acid

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a coloured product which remained in the aqueous solution during ether extraction and moved fast on a 66%-methyl ethyl ketone-ether column. Their behaviour on other columns distinguishes these breakdown products from the DNP-amino-acids. No other DNP-amino-acids give rise to 'artefact' bands with R_F values less than 1·0 on a chloroform column, though some of them give a band moving fast which is probably 2:4-dinitroaniline.

Table 2. *Approximate breakdown of DNP-amino-acids on acid hydrolysis*

	Hydrolysis in boiling 5·7 N-HCl		Hydrolysis for 16 hr. in 12 N-HCl at 105° (minimal figures).
Amino-acid derivative	Time of hydrolysis (hr.)	Amount unchanged (%)	Amount unchanged (%)
DNP-alanine	12	80	75
DNP-arginine	12	90	75
DNP-aspartic acid	24	60	75
bis-DNP-cystine	12	25	0
DNP-glutamic acid	12	75	75
DNP-glycine	8	40	50
DNP-hydroxyproline	4	40	50
DNP-isoleucine	12	80	75
DNP-leucine	12	80	75
bis-DNP-lysine	8	95	75
ε-DNP-lysine	12	95	75
DNP-methionine	12	75	75
DNP-phenylalanine	12	70	50
DNP-proline	2	10	50
DNP-serine	12	90	75
DNP-threonine	24	90	75
DNP-tryptophan	12	90	0
DNP-tyrosine	12	75	50
DNP-valine	12	80	75

Hydrolysis in approx. 12 N-HCl *at* 100°. Many conditions of hydrolysis were investigated in order to find a method suitable for the estimation of DNP-proline. It was found that if DNP-proline and globin were heated in a sealed glass tube with 12 N-HCl at 105° for 16 hr., less than 50% decomposition was produced. The stability of DNP-hydroxyproline at the higher acidity was greater than in boiling 5·7 N-HCl, but with a number of other DNP-amino-acids (e.g. tryptophan and cystine) the decomposition was greatly increased. Earlier observations had suggested that proline might be a terminal amino-acid in salmine, and hence this protein was used to test the efficiency of the method of hydrolysis. Using 12 N-HCl in a sealed tube we were able to confirm that proline is in fact the only terminal amino-acid of salmine. This was expected from the inability of salmine to yield nitrogen in the Van Slyke apparatus. The great lability of DNP-proline had not been observed during the earlier experiments carried out on insulin (Sanger, 1945) and gramicidin S (Sanger, 1946). Using the above method of hydrolysis we have been able to confirm that in neither is proline a terminal residue.

Reaction of FDNB with valine

In order to test whether the reaction with valine is complete in 2 hr., two samples of DL-valine were allowed to react with FDNB for 2 and 16 hr. respectively, the DNP-valine being estimated after purification on a chloroform column. The results obtained are shown in Table 3.

Table 3. *Reaction of FDNB with valine*

Time of reaction (hr.)	Yield (mg. α-amino N)
2	0·22
16	0·24
Calculated	0·225

The discrepancy is close to the experimental error and is not considered significant. In order to ensure that the time necessary for the reaction was not increased when the amino-acid was in combination in a protein, the effect of increasing the time of reaction of FDNB with haemoglobin from 2 to 24 hr. was investigated. Again the quantitative results showed no significant difference.

Preparation of the DNP-globins

Horse haemoglobin was prepared by the method of Adair & Adair (1934). A similar method was used for goat and donkey haemoglobins, but crystallization was induced by dialysis against ammonium sulphate solution of suitable concentration. The cow, sheep and human haemoglobins were prepared by the method of Boor & Hetkoen (1930), human foetal haemoglobin from the blood of a 30-week foetus by the method of Haurowitz (1935), and horse myoglobin by the method of Theorell (1932).

The DNP-globins were prepared as described for DNP-insulin (Sanger, 1945), but two types of preparation were made. In the first the haem was removed (Anson & Mirsky, 1934) before the reaction with FDNB, and in the second afterwards. This was done in order to determine if the coupling of the haem with the globin blocked any of the free amino groups. The amount of globin in the air-dried DNP-globins was calculated from the amide content as previously described. The values are shown in Table 4.

Table 4. *Globin content of air-dried DNP-globins*

DNP-globin	Globin (%)
Cow	72
Donkey	75
Goat	75
Horse	76
Human adult	77
Human foetal	80
Sheep	70
Horse myoglobin	70

Measurement of the DNP-amino-acids

The DNP-amino-acids were measured colorimetrically with a photoelectric absorptiometer after solution in N-HCl or 1% (w/v) $NaHCO_3$. Contrary to a previous statement it was found that the colorimetric curves obtained with the DNP-derivatives of the L- and DL-amino-acids studied were identical. The losses of DNP-valine and DNP-methionine during hydrolysis in the presence of globin are shown in Table 5.

Table 5. *Breakdown of DNP-amino-acids on acid hydrolysis*

Method of hydrolysis	DNP-amino-acid	Time of hydrolysis (hr.)	Amount unchanged (%)
Boiling 5·7 N-HCl	DNP-valine	16	75
		24	64
	DNP-methionine	8	85
		24	46
12 N-HCl at 105°	DNP-valine	16	90
		40	75

As the factor which is used to correct for breakdown of DNP-amino-acids during hydrolysis is probably the principal source of error, the time of hydrolysis was always reduced until peptides were detected. In the globins about 10% of DNP-valine was still in peptide form after 16 hr. hydrolysis with 5·7 N-HCl whilst only a trace of a methionyl peptide was detected after 8 hr. hydrolysis. DNP-valyl peptides could just be detected after 40 hr. hydrolysis in 12 N-HCl at 100°. It is interesting to note that the intensity of the colour of DNP-valine and DNP-glycine is considerably reduced when they are in peptide form. Hence, peptides, if present, were separated on the columns, hydrolyzed further and estimated separately.

RESULTS

Identification of the terminal residues in haemoglobins

In human adult, human foetal, horse and donkey haemoglobins only one terminal amino-acid was found. By passing the DNP-derivative obtained from the hydrolysate through a number of different columns and comparing the R_F values with that of synthetic DNP-amino-acids, the terminal residue was found to be valine. This was confirmed by showing that the DNP-derivative could be separated on the appropriate column from all other DNP-amino-acids except DNP-valine. In a similar manner it was demonstrated that cow, sheep and goat haemoglobins contain two terminal residues which were identified as valine and methionine. Glycine was found to be the only terminal amino-acid in horse myoglobin.

Estimation of the terminal residues in haemoglobins

The results obtained are listed in Table 6. With the exception of the human foetal haemoglobin the figures for the numbers of the free amino groups

Table 6. *The numbers of free amino groups of haemoglobins*

		No. of free α-amino groups/mol. of mol. wt. 64,000		No. of free lysine ϵ-NH_2 groups/mol. wt. of 64,000
Globin	Conditions of hydrolysis	Valine	Methionine	
Horse	24 hr. boiling 5·7 N-HCl	6·3, 6·1, 5·8	Nil	42, 41, 40
	16 hr. boiling 5·7 N-HCl	5·9	—	—
	40 hr. 100° 12 N-HCl	6·0	—	—
	Mean	6·0	Nil	41
Donkey	16 hr. boiling 5·7 N-HCl	5·6, 6·0	Nil	40, 42
	Mean	5·8	Nil	41
Cow	24 hr. boiling 5·7 N-HCl	2·2, 2·0	1·9, 2·3	46, 48
	Mean	2·1	2·1	47
Goat	16 hr. boiling 5·7 N-HCl	2·4	1·8	47, 49
	8 hr. boiling 5·7 N-HCl	2·3, 2·0	1·7, 1·8	—
	Mean	2·3	1·8	48
Sheep	24 hr. boiling 5·7 N-HCl	2·1, 2·1, 2·0	2·0, 1·9, 1·9	46, 48, 48
	8 hr. boiling 5·7 N-HCl	2·1	2·2	—
	40 hr. 100° 12 N-HCl	2·3	—	—
	Mean	2·1	2·0	47
Human adult	24 hr. boiling 5·7 N-HCl	5·1, 4·8, 5·3	Nil	42, 43, 45
	16 hr. boiling 5·7 N-HCl	4·8	—	—
	40 hr. 100° 12 N-HCl	5·2	—	—
	Mean	5·1	—	43
Human foetal	24 hr. boiling 5·7 N-HCl	2·5, 2·6, 2·7	Nil	47, 45, 50
	Mean	2·6	—	47
		Glycine/mol. wt. 17,000		Lysine/mol. wt. 17,000
Horse myoglobin	4 hr. boiling 5·7 N-HCl	1·2, 1·2, 1·3		17, 21, 24
	2 hr. boiling 5·7 N-HCl	0·9, 1·1		20
	8 hr. 100° 12 N-HCl	0·9		—
	Mean	1·1		20

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Table 7. *Summary of results*

Haemoglobin	Assumed mol. wt.	Terminal residues	Free lysine ε-amino groups
Horse	66,000	6 valine	41
Donkey	66,000	6 valine	41
Human adult	66,000	5 valine	43
Cow	66,000	2 valine, 2 methionine	47
Sheep	66,000	2 valine, 2 methionine	47
Goat	66,000	2 valine, 2 methionine	48
Human foetal	66,000	2·6 valine	47
Horse myoglobin	17,000	1 glycine	20

have been approximated to the nearest whole number and are summarized in Table 7.

No accurate figure is yet available for the molecular weight of human foetal haemoglobin. The sedimentation constant (Andersch, Wilson & Menten, 1944) suggests a molecular weight about half that of the adult haemoglobin, but as the diffusion constant was not measured the true value could not be calculated. The results obtained would suggest one terminal residue/molecule of mol.wt. 25,000 or two/molecule of mol.wt. 50,000.

The accuracy of the figures obtained is of particular importance in assessing the significance of the difference between the six terminal valyl residues found in horse haemoglobin and the five found in human haemoglobin. When a breakdown correction of 25% is used there must be doubt as to the importance of a difference of only 15–20%; however, the consistency of the figures obtained under a variety of conditions of hydrolysis and the absence of any overlapping justifies, it is felt, the conclusion that there is a structural difference between the two proteins.

DISCUSSION

The significance of the results will be discussed, first, as to the light they can throw on the molecular structure of the haemoglobins, and secondly, as to the correlation that can be found between the structural pattern and the immunological specificity of the molecule.

Structure. The number of free α-amino groups gives the number of open peptide chains present in the protein molecule. Possibly there also exist in the molecule cyclic chains or chains branching off the ω-carboxyl groups of glutamic or aspartic acid. Such chains would contain no free α-amino groups. The presence of cyclic chains has been demonstrated in gramicidin S (Sanger, 1946) and tyrocidine (Christensen, 1945) and thus may occur in proteins. However, in the absence of further evidence, the simplified assumption will be made in the following discussion that the number of open peptide chains is equal to the total number of chains in the molecule. Even if this is not true, it is likely that the number of open chains has a similar significance in relation to structure and immunological specificity.

The marked structural difference between the human adult and foetal haemoglobins was to be expected in view of the other differences that have been observed, e.g. rate of alkali denaturation (Brinkman & Tonxis, 1935), immunological specificity (Darrow, Nowakovsky & Austin, 1940), electrophoretic mobility and sedimentation rate (Andersch *et al.* 1944). It would seem clear that the difference in the two molecules arises from dissimilarity of chemical structure, and not from a physical association or dissociation of the same chemical units.

It is also apparent from lysine content, terminal amino-acid residue, molecular weight and other properties that myoglobin is chemically quite distinct from haemoglobin of the same species.

Of the normal haemoglobins, those from the cow, sheep and goat have four chains/molecule, two with a terminal valyl residue and two with a terminal methionyl residue. Roche, Michel & Schiller (1944) claim to have isolated the phenyl hydantoin of phenylalanine from a hydrolysate of sheep globin which had been treated with phenyl *iso*cyanate, but we could find no evidence of a terminal phenylalanyl residue. The human haemoglobin has five chains, each with a terminal valyl residue, and the horse and donkey haemoglobins six chains with terminal valyl residues. Boyes-Watson & Perutz (1943), from an X-ray study of a single crystal of horse haemoglobin, deduced that the molecule probably consisted 'of four equal and parallel layers of scattering matter'. This need not conflict with our results as the lengths of the six chains are not known, and it is possible that they could be grouped or folded in such a manner as to give four such equal layers.

The results given in Table 6 were obtained on preparations of DNP-globin from which the haem had been removed either before or after the reaction with FDNB. No difference was found in the numbers of the terminal amino-acids, thus proving that the haem is not linked to the globin through an α-amino group. The accuracy of the method is such that a difference of one or two in the number of free lysine ε-amino groups could not be detected with certainty. The possibility of the linkage of the haem through this group must therefore remain.

The lysine contents of three of the haemoglobins used have been determined by Dr Macpherson in this laboratory and are given in Table 8. Comparison of the number of free ε-amino groups as measured by this method and the lysine contents shows good agreement. It is therefore probable that all these groups are free in these proteins, and that no branching or cross linking of the peptide chains occurs through them.

Table 8. *Lysine contents of haemoglobins*

Haemoglobin	Mol. wt.	Number of lysine residues/mol.	Number of free lysine ε-amino groups/mol.
Horse	66,000	39	40
Sheep	66,000	45	47
Horse myoglobin	17,000	19	20

Relation of molecular structure to immunological specificity. An extensive study of the immunological behaviour of the haemoglobins has been made by Hetkoen & Schulhof (1923), Hetkoen & Boor (1931) and Heidelberger & Landsteiner (1923). The results show that in their serological properties the haemoglobins are similar to the other pure proteins that have been examined, and the conclusions may be summarized as follows: (1) Proteins having the same function in genealogically related animals cross-react with each other. (2) The serological differences between such proteins increase with the distance apart in the zoological scale, but some similarity persists even in proteins from distantly related animals. (3) The specificity of a protein is altered by certain types of denaturation, but some species specificity remains.

Our results are in parallel with the first conclusion; the haemoglobins from the horse and donkey, which cross react strongly, both have the same terminal residues and number of open chains. Those from the cow, sheep and goat fall into another group, both by end-group assay and immunological behaviour, whereas the human adult and foetal haemoglobins are distinct from each other and from the first two groups in both respects. With regard to the second conclusion it is interesting to note that all the haemoglobins examined have at least one chain with a terminal valyl residue, but myoglobin, shown by Hetkoen (1928) to be immunologically distinct from haemoglobin in the dog, has one chain with a terminal glycyl residue. It should be noted that its molecular weight and amino-acid composition are also markedly different.

Landsteiner's study of azo proteins originated the idea of small determinant groups which he designated haptenes. According to this concept certain polar groups in the native protein molecule would function as haptenes and the specificity of the molecule would be the result of their net action. The effect of denaturation by heat, dilute acid or dilute alkali (Macpherson & Heidelberger, 1945), contrary to denaturation by urea or guanidine (Erickson & Neurath, 1943), produces a drastic alteration in the specificity of the protein. This presumably implies that the configuration or folding of the peptide chains within the molecule plays an important part in determining the specificity. This apparently conflicting evidence can best be reconciled by assuming that the haptene-functioning parts of the molecule are influenced by the configuration of the molecule. The determinant groups may arise by the folding of the chain to give a specific array of polar side-chain residues which in the unfolded chain are distantly spaced. Such an array would be dependent on the configuration of the molecule. The disordering of the polar groups, as might occur in certain types of denaturation, would disrupt the specificity-determining parts of the molecule and would lead to a marked change in immunological behaviour. In such a completely denatured protein, where the configuration is believed to be random, the specificity would then depend only on the relative numbers of the different amino-acids and their order along the peptide chain. The haemoglobins of allied species have been shown to have a similar number of open peptide chains and probably have a similar amino-acid composition. It would thus be expected that denaturation would increase the cross reactions of the haemoglobins from related species whilst the distinction between haemoglobins with different numbers of chains/molecule would be maintained. Evidence on this point could not be found in the literature and it is hoped to investigate this further.

Our results suggest a relationship between the terminal residues and immunological specificity. That the free amino groups are part of the determinant groups seems unlikely from the work with toxins and toxoids (Pappenheimer, 1938), and it seems probable that the number of chains in the molecule plays a more predominant role in the immunological behaviour, and this suggests the importance of gross structure in addition to amino-acid composition in determining the specificity of a protein. Our results bring out chemical similarities as well as differences between the haemoglobins, a point not previously shown. The immunological differences that exist between closely allied species can no doubt be accounted for by slight variations in configuration. The results are therefore in agreement with the suggestion as to the basis of the specificity of native proteins outlined above, but much more evidence will be required before more definite conclusions can be drawn.

SUMMARY

1. Several DNP-amino-acids have been prepared and characterized.

2. Several modifications of the method of measuring the free amino groups of proteins with 1:2:4-fluorodinitrobenzene are described.

3. The method has been applied to several haemoglobins. Horse and donkey haemoglobins contain six terminal valyl residues/molecule, adult human haemoglobin five terminal valyl residues, and cow, sheep and goat haemoglobins two terminal valyl and two terminal methionyl residues. If a molecular weight of 66,000 is assumed, human foetal haemoglobin contains 2·6 terminal valyl residues. Horse myoglobin has one terminal glycyl residue/molecule of molecular weight 17,000. The ϵ-amino groups of the lysine residues are free in the above proteins.

4. The significance of these results in relation to the structure of the haemoglobins and the correlation between structure and immunological specificity of proteins is discussed.

We wish to express our thanks to Miss J. Keilin for preparing the horse myoglobin, to Dr H. T. Macpherson for the lysine analyses reported in Table 8 and to Prof. A. C. Chibnall for his advice and encouragement.

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Metabolism of Acetoacetate in Animal Tissues. 2

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The first paper on this subject (Krebs & Eggleston, 1945) was concerned with the anaerobic reactions of acetoacetate. It was shown that in isolated sheep heart two rapid reactions of acetoacetate occur:

acetoacetate + L-malate
= L-β-hydroxybutyrate + oxaloacetate,
acetoacetate + α-ketoglutarate
= L-β-hydroxybutyrate + succinate + CO_2.

These two reactions account for the *anaerobic* metabolism of acetoacetate in sheep heart muscle: all the acetoacetate removed was recovered, within the limits of error, as β-hydroxybutyrate. The present paper deals with the *aerobic* reactions of acetoacetate. Whilst this work was in progress some of its problems were solved by Buchanan, Sakami, Gurin & Wilson (1945) with the help of isotopic carbon. These workers showed beyond doubt that the carbon skeleton of acetoacetate enters the tricarboxylic cycle.

The main contributions of the present paper are measurements of the rate of the oxidation of acetoacetate in various tissues and the effect on these rates of the physiologically occurring dicarboxylic acids, as well as quantitative estimations of the products of acetoacetate metabolism. The results are in agreement with the conception that acetoacetate is oxidized through the tricarboxylic cycle.

METHODS

Tissue preparations

Suspensions of minced tissue were used in the same way as in the previous experiments (Krebs & Eggleston, 1945), unless otherwise stated. The metabolic activities were interrupted after a specified period of incubation by addition of 0·25 vol. of 2N-HCl.

Analytical procedures

Changes in the previous methods concerned the following points.

Determination of pyruvate in the presence of oxaloacetate. Oxaloacetate was not found in significant quantities when heart or kidney were incubated with this substrate under anaerobic conditions (unless the period of incubation was very short). Aerobically, however, oxaloacetate was often present, presumably because it was continuously reformed from malate. The problem therefore arose of determining pyruvate in the presence of oxaloacetate. Carboxylase decarboxylates oxaloacetate under the conditions used for the determination of pyruvate, but the amounts of CO_2 split off are variable. Under the standard conditions for the determination of pyruvate the initial rate of CO_2 formation from oxaloacetate was about 15% of that from pyruvate. The rate of CO_2 production gradually slowed down and the reaction practically stopped after 6 hr. when about 1·2 moles CO_2 had been formed/mole oxaloacetate (Table 1). The slowing down of the rate was not due to inactivation of carboxylase, for addition of pyruvate showed that the enzyme was still highly active. In more concentrated yeast extracts (4 ml. yeast extract, 0·2 ml. 3M-acetate buffer, pH 5·0; 0·1 ml. 0·1M-substrate solution) the total yield of CO_2 from oxaloacetate was only 0·6 mole/mole. These observations indicate that there are other reactions of oxaloacetate besides decarboxylation, the extent of which varies with the conditions. Neuberg & Karczag (1911) found that their yeast suspension liberated 2 moles of CO_2 from 1 mole oxaloacetate.

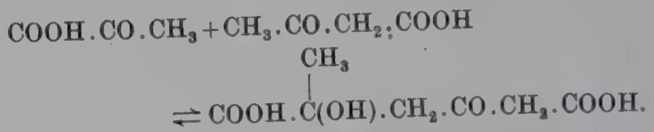
Table 1. *Decarboxylation of oxaloacetate by yeast extract*

(Yeast extract from English brewer's yeast (see Krebs & Johnson, 1937). Conditions for the manometric determination of CO_2 evolution according to Krebs & Eggleston (1945). The main compartment of each cup contained 3 ml. water; 0·1 ml. 0·1M-substrate; 0·2 ml. 3M-acetate buffer, pH 5·0; side arm, 1 ml. carboxylase. The data given are corrected for a small CO_2 evolution from yeast without substrate.)

	Substrate	
Time after carboxylase addition (min.)	Pyruvate (224 μl.)	Oxaloacetate (224 μl.)
	CO_2 evolved (μl.)	
2	84	11·5
4	160·5	25·5
8	217·5	61·5
12	224	89
20	224	136
40	224	211
120	227	251
180	—	259
240	—	269

The following procedure was proved satisfactory in overcoming the difficulties created by the irregular yields of CO_2 from oxaloacetate: A measured fraction of the acidified tissue suspension containing 0·4N-HCl was heated for 15 min. in a water bath at 100° and the pH was then adjusted to 5·0 by addition of 3M-sodium acetate. This treatment converts oxaloacetate quantitatively into pyruvate and CO_2 whilst not destroying preformed pyruvate. The determination of pyruvate in the solution thus yielded the sum of pyruvate and oxaloacetate. In another fraction of the tissue suspension oxaloacetate was determined by the aluminium sulphate method (Krebs & Eggleston, 1945). The difference represented the preformed pyruvate.

Determination of oxaloacetate in the presence of pyruvate and acetoacetate. The manometric method previously used was based on the observation that multivalent cations specifically decarboxylate β-keto-dicarboxylic acids (Krebs, 1942). In the course of the present work it was found that acetoacetate reacts also if pyruvate or other α-ketonic acids are present (Table 2). In other words, α-ketonic acids have a catalytic effect on the decomposition of acetoacetate by aluminium ions. It is very probable that this is due to the formation, by aldol condensation, of a dicarboxylic β-ketonic acid from the α-ketonic acid and acetoacetate, e.g.



$$\mathrm{COOH.CO.CH_3 + CH_3.CO.CH_2.COOH \rightleftharpoons COOH.\overset{\displaystyle CH_3}{\overset{|}{C}}(OH).CH_2.CO.CH_2.COOH.}$$

In general the effect of α-ketonic acids was not great because of the low concentration of these substances in the solutions to be tested. The magnitude of the effect was estimated by extrapolation. Warburg cups were filled as previously described for the determination of oxaloacetate, and readings were taken for a period of 60 min. after the addition of $Al_2(SO_4)_3$. Under the conditions of the test oxaloacetate was quantitatively decomposed after 40 min. The CO_2 evolution which occurred between 40 and 60 min. was taken as due to the decomposition of acetoacetate and twice this value was deducted from the 40 min. reading to obtain the amount of oxaloacetate in the solution. This correction factor was probably slightly too high as the rate of CO_2 evolution from mixtures of acetoacetate and α-ketonic acid slightly increased with time (Table 2).

Acetoacetate does not seem to form appreciable quantities of such a condensation product with a second molecule of acetoacetate as long as the concentration of acetoacetate is low (0·005M). At higher concentrations (0·25M final concentration) Al ions accelerate the decarboxylation: without Al^{+++}, 0·25% was decomposed in 60 min., and with Al^{+++} 2·1% (conditions as stated in Table 2).

Table 2. *Evolution of CO_2 from mixtures of acetoacetate with α-ketonic acids in the presence of aluminium salts*

(Conditions as given by Krebs & Eggleston (1945) for the determination of oxaloacetate. Each cup contained 0·2 ml. 0·1 M-acetoacetate in addition to the substrate stated below. Concentration of added substrate solutions 0·1 M.)

	Substrates added						
Time after Al^{+++} addition (min.)	0	0·05 ml. pyruvate	0·2 ml. pyruvate	1·0 ml. pyruvate	0·05 ml. α-keto-glutarate	0·1 ml. α-keto-glutarate	0·2 ml. α-keto-glutarate
	CO_2 evolved (μl.)						
10	0	0	19	80	0	7	32
20	0	5	44	147	0	14	64
40	0	17	93	232	0	29	123
60	0	26	126	272	3·5	46·5	165
120	4	62	188	320	17	88·5	249
240	14	86	239	343	—	—	—

Table 3. *Substances simulating β-hydroxybutyrate in the method of Van Slyke* (1917) *and of Edson* (1935)

Substance	Amount added (mg.)	'β-Hydroxybutyrate' formed (mg.)	Yield (mg. β-hydroxy-butyrate/100 mg. substance tested)
L-Lactic acid	18	6·31	35·1
	9	2·80	31·1
	4·5	0·99	22·0
	2·25	0·04	1·78
	1·125	0·04	3·56
L-Malic acid	26·8	2·03	7·6
	13·4	1·14	8·5
	6·7	0·21	3·1
Fumaric acid	23·2	4·65	20·0
	11·6	1·80	15·5
	5·8	0·48	8·3
α-Ketoglutaric acid	29·2	0·287	0·98
	14·6	0·093	0·61
	7·3	0·032	0·23
Pyruvic acid	17·6	0·505	2·87
Citric acid	38·4	0·884	2·3
Succinic acid	23·6	Nil	Nil

Determination of β-hydroxybutyrate. The method previously used (Edson, 1935; Krebs & Eggleston, 1945) was modified in the following points in order to increase accuracy. Instead of 1 ml. 5% potassium dichromate, 2·5 ml. were added. The precipitate formed after the dichromate oxidation was filtered off when the solution had cooled to room temperature. Sintered glass funnels (3 G 3) allowing about 5–7 ml. fluid to filter/min. were used. Under the conditions of the determination 1·0 ml. 0·01 N-iodine were equivalent to 0·166 mg. β-hydroxybutyric acid in the filtrate.

Several substances present in the solution were found to simulate small quantities of β-hydroxybutyrate. Van Slyke (1917, 1929) already found this for lactate provided it was present in fairly large quantities. Data on the errors caused by various substances are given in Table 3. The concentrations of interfering substances were known and the data in Table 3 could thus be used for correcting results. The correcting factor was generally below 5% of the amounts of β-hydroxybutyrate as the quantities of the interfering substances in the sample were small, viz. lactic acid between 0·3 and 1·2 mg.; malic acid between 0·5 and 2 mg.; fumaric acid between 0·5 and 1 mg.

The errors were of the same order in pure solutions and in mixtures with β-hydroxybutyrate. Attempts to increase the specificity of the method by separating β-hydroxybutyrate from the interfering substances through chromatography were unsuccessful.

Fumaric acid was in a number of cases determined by the manganometric method of Straub (1935).

Citric acid. Water was used instead of dioxan to dilute the final alkaline Na_2S solution to the required volume in the colorimetric determination. The colour was found to be more stable in this medium than in aqueous solutions of commercial 'pure' dioxan.

RESULTS

Comparative rates of reduction and oxidation of acetoacetate

In Table 4, experiments on sheep heart and sheep kidney cortex are recorded in which the amounts of acetoacetate removed and β-hydroxybutyrate

Table 4. *Amounts of acetoacetate removed*

(All data refer to 4 ml. suspension; acetoacetate 'oxidized' refers to the amounts removed which are not accounted for by the formation of β-hydroxybutyrate.)

Exp. no.	Tissue and medium	Acetoacetate added (μl.)	Other substrates added (μl.)	Period of incubation (min.)	Changes in N_2			Changes in O_2		
					Acetoacetate removed (μl.)	β-Hydroxy-butyrate formed (μl.)	Acetoacetate 'oxidized' (μl.)	Acetoacetate removed (μl.)	β-Hydroxy butyrate formed (μl.)	Acetoacetate 'oxidized' (μl.)
1	Sheep heart, minced 1 part + 9 parts phosphate saline	1692	—	60	—	—	—	910	527	383
		1692	Oxaloacetate (224)	60	—	—	—	1110	646	464
		1692	Oxaloacetate (112)	60	—	—	—	1032	580	452
2	Ditto	1760	—	60	377	331	46	1062	782	280
		1760	Fumarate (448)	60	1215	1218	~Nil	1245	1040	205
3	Ditto	846	—	40	—	—	—	435	406	29
		846	Fumarate (896)	40	—	—	—	656	620	36
		846	Oxaloacetate (896)	40	—	—	—	604	561	43
4	Ditto	850	—	20	—	—	—	310	304	6
		850	—	40	—	—	—	493	349	144
		850	—	120	—	—	—	492	184	308
		850	Oxaloacetate (896)	20	—	—	—	569	375	194
		850	Oxaloacetate (896)	40	—	—	—	764	409	355
		850	Oxaloacetate (896)	120	—	—	—	691	156	535
5	Ditto	878	—	40	—	—	—	558	325	233
		878	Oxaloacetate (896)	40	—	—	—	691	417	274
		878	Fumarate (896)	40	—	—	—	706	427	279
6	Ditto	1740	—	30	—	—	—	410	304	106
		1740	Oxaloacetate (896)	30	—	—	—	825	521	304
		1740	Fumarate (896)	30	—	—	—	759	573	186
7	Sheep kidney cortex minced 1 part + 9 parts phosphate saline	1856	—	40	386	366	20	653	227	426
		1856	Fumarate (896)	40	751	778	~Nil	656	309	347
		1856	Oxaloacetate (896)	40	651	485	166	656	279	377
8	Ditto	1760	—	60	360	322	38	355	175	180
		1760	Fumarate (448)	60	680	658	22	550	320	230
9	Ditto	872	—	60	—	—	—	394	182	212
		872	Fumarate (896)	60	—	—	—	642	466	176
		872	Oxaloacetate (896)	60	—	—	—	604	366	238
10	Ditto	829	—	60	—	—	—	204	127	77
		829	Fumarate (896)	60	—	—	—	359	289	70
		829	Oxaloacetate (896)	60	—	—	—	341	158	183
11	Ditto	1285	—	90	—	—	—	647	229	418
		1285	Oxaloacetate (1344)	90	—	—	—	1084	509	575
12	Ditto	1647	—	30	—	—	—	220	161	59
		1647	Oxaloacetate (1792)	30	—	—	—	622	287	335
13	Ditto	847	Oxaloacetate (896)	20	—	—	—	261	181	80
		847	Oxaloacetate (896)	40	—	—	—	436	274	162
		847	Oxaloacetate (896)	80	—	—	—	613	239	374
		847	Oxaloacetate (896)	160	—	—	—	630	212	418
14	Ditto	1871	—	40	374	236	138	—	—	—
		1871	Oxaloacetate (1792)	40	661	436	225	—	—	—
15	Ditto	942	Oxaloacetate (448)	30	244	183	61	324	164	160
16	Sheep kidney cortex minced 1 part + 2·6 parts NaCl-$NaHCO_3$ (medium of Breusch, 1943*b*)	888	Oxaloacetate (896)	20	588	421	167	583	433	150
17	Ditto	848	Oxaloacetate (1792)	30	673	501	172	—	—	—
		848	Oxaloacetate (1792) + pyruvate (820)	30	704	488	216	—	—	—
18	Ditto	1742	Oxaloacetate (3584)	30	1406	1081	325	—	—	—
		1742	Oxaloacetate (3584) + pyruvate (1650)	30	1406	1046	360	—	—	—
19	Sheep kidney cortex minced 1 part + 7 parts phosphate-$NaHCO_3$ saline	890	Oxaloacetate (1792)	15	357	283	74	—	—	—
		890	Oxaloacetate (1792)	30	490	438	52	—	—	—
		890	Oxaloacetate (1792)	60	603	620	Nil	—	—	—

formed are compared. The data indicate the following:

(1) Anaerobically, as in the previous experiments (Krebs & Eggleston, 1945), almost all acetoacetate removed in sheep heart was accounted for by the formation of β-hydroxybutyrate. A small excess was sometimes found when no additional substrate was added, but this was probably due to the non-enzymic ketone decomposition. No correction was applied for ketone decomposition, and as the average acetoacetate concentration during the experiment was usually higher when no further substrate was added (because the reduction was slower), the error in the calculation of the amounts 'oxidized' arising from ketone decomposition was larger in the absence of additional substrates. In sheep kidney the quantities of acetoacetate 'oxidized' anaerobically exceeded the experimental error if oxaloacetate was present (Exp. nos. 7, 14, 16, 17, 18).

(2) Aerobically a large proportion of the acetoacetate removed was still due to reduction to β-hydroxybutyrate, especially in short experiments in accordance with results published by Quastel & Wheatley (1935). On prolonged incubation, however, the amount of 'oxidized' acetoacetate gradually increased, whilst the amounts of β-hydroxybutyrate, after a rapid increase during the early periods of incubation, showed a fall in the later periods. It thus appears that in the early stages, when the acetoacetate concentration is high, the system acetoacetate $\rightleftharpoons$ β-hydroxybutyrate reacts predominantly from left to right. Later, when most of the added acetoacetate has disappeared the reverse reaction predominates (Exps. 4 and 13).

(3) The amounts of acetoacetate oxidized aerobically were increased in many experiments by oxaloacetate or fumarate, both in heart muscle and in kidney. This may be taken as supporting the view that the four-carbon dicarboxylic acids play some part in the oxidation of acetoacetate (Quastel & Wheatley, 1935; Edson & Leloir, 1936; Breusch, 1943*a*, *b*; Wieland & Rosenthal, 1943; Lehninger, 1946), a conclusion which is not invalidated by the fact that the effects did not occur in every experiment (since the tissues are known to contain considerable quantities of precursors of oxaloacetate).

(4) In heart muscle, the initial, that is maximum, rate of aerobic reduction of acetoacetate calculated in terms of $Q_{\beta\text{-hydroxybutyrate}}$, assuming the dry weight to be 1/5 of the wet weight, was 11·4 without oxaloacetate, 14·1 with oxaloacetate (Table 4, Exp. 3). The maximum rates of oxidation without oxaloacetate (same exp., period 20–40 min.) in heart were 5·2 and 7·3 with oxaloacetate. In this tissue the maximum rates of reduction were thus about twice those of oxidation. In kidney cortex the corresponding rates of reduction were 4·0 without and 7·2 with oxaloacetate, the rates of oxidation 1·5 without and 8·4 with oxaloacetate (Table 4, Exp. 12). In kidney the maximum rates of oxidation and reduction in the presence of oxaloacetate therefore were of the same order.

Products of acetoacetate removal

Examples giving comprehensive analyses of the products of acetoacetate metabolism are shown in Table 5. The comparison of the yields of the various substances on the addition of oxaloacetate, with those found on addition of both oxaloacetate and acetoacetate, shows that acetoacetate caused an increase in the yields of citrate and of succinate,

Table 5. *Acetoacetate and oxaloacetate in sheep kidney*

(48 ml. suspension containing 1 part minced kidney cortex and 9 parts phosphate saline pH 7·4. All quantities are in μl. and refer to 48 ml. suspension. The three experiments were carried out on different kidneys.)

	Exp. 1 N_2 (40 min. incubation)			Exp. 2 O_2 (30 min. incubation)			Exp. 3 O_2 (30 min. incubation)	
Substrates added:								
Acetoacetate	22,460	—	22,460	19,760	—	19,760	—	12,140
Oxaloacetate	—	21,500	21,500	—	21,500	21,500	21,500	21,500
Substances found after incubation:								
Acetoacetate	17,970	~0	14,520	17,360	—	12,300	~0	6,230
β-Hydroxybutyrate	2,830	~0	5,210	1,670	—	3.410	~0	2,915
Oxaloacetate	~0	1,210	1,850	—	2,670	4,400	1,640	2,770
Pyruvate	~0	4,070	4,750	—	—	—	550	1,400
Succinate	~0	1.270	2,005	—	320	1,360	240	342
α-Keto-(plus α-hydroxy)-glutarate	1,110	1,605	1,655	1,275	2,160	550	725	~0
Fumarate	—	2,765	880	—	—	—	—	—
L-Malate	~0	4,605	3,155	—	3,640	3,240	2,590	2,500
Citrate	10	156	1,690	—	1,530	3,240	1,740	3,820
Calculated changes:								
Acetoacetate reduced	2,830	—	5,210	1,670	—	3.410	—	2.915
Acetoacetate 'oxidized'	1,660	—	2,730	730	—	4,050	—	2,995

whilst the yields of α-keto(plus α-hydroxy)glutarate were decreased. On balance, less oxaloacetate disappeared when acetoacetate was present. The effects of oxygen on the yields were altogether not very great, the most notable difference being the higher yield of citrate in O_2.

The observations on the differences in O_2 and N_2 are in general in accordance with the view that aerobically the same reactions occur as anaerobically with certain additional reactions, namely, some of the oxidative steps of the tricarboxylic cycle and the oxidation of β-hydroxybutyrate to acetoacetate. The experiments do not allow any conclusions on the mechanism of the formation of citrate in the presence of oxaloacetate and acetoacetate.

A number of similar experiments were carried out (before the tracer work of Buchanan *et al.* (1945) decided the question conclusively) with the intention of throwing light on the mechanism of the increased citric acid formation in the presence of oxaloacetate and acetoacetate, especially on the conflicting views expressed by Breusch (1943*a*, *b*) and by Wieland & Rosenthal (1943) on the one hand and by ourselves (Krebs & Eggleston, 1945) on the other. If our earlier view that acetoacetate does not take a direct part in the formation of citrate were to apply also to aerobic conditions, the sum of the reaction products arising from oxaloacetate, i.e. the acids of the tricarboxylic cycle, including pyruvic acid, would be limited by the amount of carbon added in the form of oxaloacetate. Each molecule of tricarboxylic acid, α-keto- and α-hydroxy-glutarate and the bulk of the succinate molecules would require 2 molecules of oxaloacetate and each molecule of fumarate, malic and pyruvate would require 1 molecule of oxaloacetate. According to Breusch and Wieland & Rosenthal, only 1 molecule of oxaloacetate would be needed for each molecule of acid because acetoacetate would provide part of the carbon skeleton of the tricarboxylic acids, of α-keto- and α-hydroxyglutarate and of part of succinate.

Calculations based on five experiments are shown in Table 6. According to the last column more 'oxaloacetate equivalents' were recovered in some experiments than were added; this discrepancy was always greater when acetoacetate was present. The latter observation argues against our previous contention and suggests that acetoacetate contributes towards the formation of the acids of the tricarboxylic cycle. The results in Table 6 however can hardly be taken as decisive, as the effects in question are relatively small and the errors of analysis and uncertainties in the calculations may be considerable; the errors are the aggregate from six analyses and the uncertainties arise from the question how much succinate is formed by reduction and how much by oxidation from oxaloacetate. In aerobic experiments, moreover, some succinate was presumably oxidized to fumarate though this reaction was inhibited during the initial stages of incubation owing to the inhibition of succinic dehydrogenase by oxaloacetate.

This line of approach was not continued when the results of the tracer work of Buchanan *et al.* (1945)

Table 6. *Comparison of the amounts of oxaloacetate added and the amounts of substances arising from oxaloacetate*

(The quantities of metabolites arising from oxaloacetate are expressed in 'oxaloacetate equivalents'. For the purpose of calculating the equivalents, the tricarboxylic acids, α-keto- and α-hydroxy-glutaric acids are taken as requiring two oxaloacetate equivalents/molecule, fumaric, malic and pyruvic acids as requiring one equivalent. Succinic acid was taken as requiring two equivalents because in the presence of acetoacetate most of the succinate is known to be formed by oxidation from α-ketoglutarate (Krebs & Eggleston, 1945). All data refer to 48 ml. minced tissue suspension containing 1 part of tissue for 9 parts of phosphate saline.)

Tissue	Substrates added (μl.)	Aerobic or anaerobic	Period of incubation (min.)	Oxaloacetate removed (μl.)	Acetoacetate 'oxidized' (μl.)	Oxaloacetate equivalents recovered (μl.)	Balance of oxaloacetate (μl.)
Sheep kidney cortex	(*a*) Oxaloacetate (21,500)	N_2	40	20,290	—	17,530	+2,760
	(*b*) Oxaloacetate (21,500) plus acetoacetate (22,460)			19,650	2,730	19,850	− 200
Sheep heart	(*a*) Oxaloacetate (21,500)	N_2	40	21,170	—	22,540	−1,370
	(*b*) Oxaloacetate (21,500) plus acetoacetate (22,750)			19,320	2,330	23,530	−4,210
Sheep kidney cortex	(*a*) Oxaloacetate (10,750)	N_2	30	10,280	—	11,480	−1,200
	(*b*) Oxaloacetate (10,750) plus acetoacetate (22,600)			9,320	1,460	10,820	−1,500
Sheep heart	(*a*) Oxaloacetate (21,500)	O_2	40	17,450	—	15,210	+2,240
	(*b*) Oxaloacetate (21,500) plus acetoacetate (22,270)			16,370	4,632	13,700	+2,670
Sheep kidney cortex	(*a*) Oxaloacetate (10,750)	O_2	30	10,480	—	9,980	+ 500
	(*b*) Oxaloacetate (10,750) plus acetoacetate (22,600)			9,850	3,850	13,090	−3,240

became available, as this work provided direct evidence for the participation of acetoacetate in the formation of the tricarboxylic acids and α-ketoglutarate. The data reported in their paper are in accordance with this conception.

Metabolism of DL-*threo- and* DL-*erythro*-1:2-*dihydroxybutyric acids in sheep kidney cortex*

If it is agreed that acetoacetate provides part of the tricarboxylic acid and α-ketoglutarate molecule, there remains the problem to be solved of the intermediary stages of the formation of these substances from oxaloacetate and acetoacetate.

The rapid reduction of acetoacetate to β-hydroxybutyrate, even in the presence of O_2, suggests the possibility that the reduction is a stage in the final breakdown of acetoacetate. This step might then be followed by an oxidation of β-hydroxybutyrate to 1:2-dihydroxybutyric acid (see Weil-Malherbe, 1938; Haarmann & Schroeder, 1938; Hoff-Jørgensen, 1940). There are apparently conflicting statements in the literature on the metabolism of 1:2-dihydroxybutyric acids. Weil-Malherbe (1938) found that kidney does not remove the erythro form, but oxidizes the threo form to α-hydroxyacetoacetate, a reaction to which he attaches no physiological significance because α-hydroxyacetoacetate is not further metabolized. Hoff-Jørgensen reported on the other hand that the erythro form is rapidly destroyed after intravenous injection, whilst the threo form was stable under the same conditions. In view of these discrepancies the question was reexamined. The results essentially confirm Weil-Malherbe's findings (Table 7). The erythro form was not attacked by sheep kidney; the threo form caused a slight increase in the O_2 uptake and a slight decrease of the concentration equivalent to 0·8 ml. 0·01 N-iodine. This is accounted for, according to Weil-Malherbe, by oxidation to α-hydroxyacetoacetate. Thus neither the threo nor the erythro forms can be intermediates in the removal of ketone bodies by sheep kidney cortex.

Table 7. *Metabolism of* DL-*threo- and* DL-*erythro*-1:2-*dihydroxybutyric acids in sheep kidney cortex*

(Minced tissue suspended in 9 parts phosphate saline. Incubation 90 min. Determination of dihydroxybutyric acid with periodate according to Weil-Malherbe (1938); the iodine equivalent of the added substrate was 16 ml. 0·01 N-iodine at the beginning; 1 ml. 0·01 N-I_2 = 112 μl. DL-dihydroxybutyrate.)

		Iodine equivalent (ml. 0·01 N-I_2) of 4 ml. suspension		
Substrate added (final conc. 0·02 M)	Q_{O_2}	At end of incubation	Corrected for 'blank'	Change during incubation
—	−12·4	5·9	—	—
Erythro form	−12·3	23·9	18·0	+2·0
Threo form	−13·0	21·1	15·2	−0·8

Sequence of formation of α-ketoglutaric and tricarboxylic acids

From Ochoa's work (1945) it is known that the reaction *iso*citrate → α-ketoglutarate + CO_2 is reversible in some tissues. Hence the system

$$\text{citrate} \underset{+H_2O}{\overset{-H_2O}{\rightleftharpoons}} cis\text{aconitate} \underset{-H_2O}{\overset{+H_2O}{\rightleftharpoons}} iso\text{citrate} \underset{+2H}{\overset{-2H}{\rightleftharpoons}} \alpha\text{-ketoglutarate} + CO_2 \qquad (1)$$

is completely reversible and the simultaneous occurrence of α-ketoglutarate and the tricarboxylic acids, as the result of an interaction between oxaloacetate and acetoacetate (or between other metabolites), may be due to the system (1) reacting from left to right, or in the reverse order (see also Wood, 1946). The following experiments were designed to throw light on this question. If the sequence is

$$\text{oxaloacetate} + \text{acetoacetate} \rightarrow \text{tricarboxylic acid} \rightarrow \alpha\text{-ketoglutarate} \qquad (2)$$

tricarboxylic acids should yield α-ketoglutarate at least as rapidly as a mixture of oxaloacetate and acetoacetate. Experiments in Table 8 show that this is the case in sheep kidney. Various substrates and substrate combinations were added to malonate-poisoned tissue, and the sum of α-ketoglutarate and succinate was measured after aerobic incubation. The reductive formation of succinate from fumarate is small under the conditions of the experiment, and the sum of α-ketoglutarate and succinate may therefore be taken as a measure of the formation of α-ketoglutarate.

It will further be seen from Table 8 that pyruvate, acetate and acetoacetate cause some formation of α-ketoglutarate. In Exp. III the increase caused by combination of pyruvate + acetate and pyruvate + acetoacetate is somewhat larger than the sum of the individual increases, but in every case the tricarboxylic acids yield α-ketoglutarate more rapidly than other substrates. The results are thus in accordance with the sequence (2). On the other hand, the following experiments exclude the sequence oxaloacetate + acetoacetate → α-ketoglutarate → tricarboxylic acid. Suspension of sheep kidney was anaerobically incubated with various substrate combinations in a medium containing 0·02 M-NaHCO$_3$ and in equilibrium with 5 % CO$_2$ in the gas phase. Table 9 shows that α-ketoglutarate yields only traces of citrate, less than 10 % of the amount formed from a combination of oxaloacetate and acetoacetate.

Table 8. *Rate of formation of α-ketoglutarate from various precursors in sheep kidney cortex*

(Minced tissue suspended in 9 parts phosphate saline pH 7·4 containing 0·005 M-malonate. O$_2$. Final substrate concentrations in Exp. 1: 0·01 M except oxaloacetate which was 0·02 M; in Exps. II–IV 0·02 M. The data refer to 4 ml. suspension; 'α-ketoglutarate + succinate' is the amount of succinate found in the solution after treatment with acid permanganate.)

		α-Ketoglutarate + succinate formed (μl.) in presence of											
Exp.	Period of incubation (min.)	Nil	Citrate	*Cis*-aconitate	Aceto-acetate	Citrate + aceto-acetate	Pyruvate	Pyruvate + aceto-acetate	Oxalo-acetate	Oxalo-acetate + aceto-acetate	Oxalo-acetate + pyruvate	Acetate	Pyruvate + acetate
	30	59	463	—	87	471	91	110	342	464	356	—	—
II	40	91	492	405	—	—	183	—	—	—	—	93	235
III	40	46	398	—	88	—	109	188	—	—	—	80	182
IV	20	90	—	—	103	—	137	147	—	—	—	—	—
	40	77	—	—	109	—	200	236	—	—	—	—	—

Table 9. *Rate of citrate formation in sheep kidney cortex from various precursors*

(Minced tissue suspended in 9 parts phosphate bicarbonate saline. 5% CO$_2$ in N$_2$. Substrate concentrations: 0·02 M except acetoacetate which was 0·01 M. Period of incubation 30 min. The data refer to 4 ml. suspension.)

Substrates added	Nil	α-Keto-glutarate	α-Keto-glutarate + aceto-acetate	Oxalo-acetate	Oxalo-acetate + aceto-acetate	Pyruvate + aceto-acetate	α-Keto-glutarate + oxalo-acetate + aceto-acetate
μl. citrate formed	Nil	23	23	119	298	23	258

Order of formation of tricarboxylic acids

It is now generally believed that the sequence of formation of the tricarboxylic acids in the tricarboxylic cycle is as follows:

$$\left.\begin{array}{c}\text{oxaloacetate}\\ +\\ \text{pyruvate (or aceto-}\\ \text{acetate, or acetate)}\end{array}\right\}\rightarrow \begin{array}{c}\textit{cis}\text{aconitate}\\ \downarrow\uparrow\\ \text{citrate}\end{array} \rightleftharpoons \begin{array}{c}\textit{iso}\text{citrate}\\ \downarrow\\ \text{oxalosuccinate}\end{array} \qquad (3)$$

This scheme rests chiefly on the work of Wood *et al.* (1941), Evans & Slotin (1941), Buchanan *et al.* (1945) and Weinhouse *et al.* (1945) which showed that citrate cannot be an intermediate on the main path leading from oxaloacetic acid to oxalosuccinic acid. Further evidence supporting this scheme is supplied by the experiments described in this section in which the formation of citrate on the one hand and of the sum of *cis*aconitate and *iso*citrate on the other was measured with the help of a procedure developed in this laboratory (Krebs & Eggleston, 1944). The interpretation of these measurements is based on a characteristic property of aconitase: if citrate arises in the presence of aconitase (from a precursor other than *cis*aconitate or *iso*citrate) 10·5 % will gradually be converted into a mixture of *cis*aconitate and *iso*citrate (Krebs & Eggleston, 1943; Martius & Leonhardt, 1943). If, however, *cis*aconitate arises according to scheme (3) a large proportion (up to 25 %) will be converted into *iso*citrate during the early stages of the reaction. In the later stages *iso*citrate (if it is not dehydrogenated) may be reconverted into *cis*aconitate and thence into citrate until an equilibrium containing 89·5 % citrate is established (Martius, 1938; Krebs & Eggleston, 1943). Therefore, (*a*) if citrate were the primary product of interaction between oxaloacetate and its partner, the sum of *cis*aconitate and *iso*citrate could never exceed 10·5 % of the tricarboxylic acids present (but may be less if the reaction is interrupted before equilibrium is established, or if *iso*citrate is dehydrogenated more rapidly than it is formed); (*b*) if *cis*aconitate is produced according to scheme (3) the sum of *cis*aconitate and *iso*citrate may exceed 10·5 % of the total tricarboxylic acids present provided that the reaction is interrupted before equilibrium is established (but may be less if *iso*citrate is dehydrogenated more rapidly than it is formed).

It follows that if the sum of *cis*aconitate and *iso*citrate is found to exceed the limit of 10·5 % this may be taken as conclusive evidence against the scheme (4) and as direct support of scheme (3).

$$\left.\begin{array}{c}\text{oxaloacetate}\\ +\\ \text{pyruvate, or}\\ \text{acetoacetate or}\\ \text{acetate}\end{array}\right\} \rightarrow \text{citrate} \rightleftharpoons \textit{cis}\text{aconitate} \rightleftharpoons \textit{iso}\text{citrate} \rightarrow \text{oxalosuccinate} \quad (4)$$

Table 10 shows the results of four experiments in which the tricarboxylic acids were estimated under varying conditions. The incubation periods were relatively short because it was intended to interrupt the reaction before an equilibrium between the three tricarboxylic acids could be established. In several experiments the amounts of *cis*aconitate + *iso*citrate far exceeded the critical figure of 10·5 %, the highest value being 49 %. The fact that the ratio

$$\frac{\textit{cis}\text{aconitate} + \textit{iso}\text{citrate}}{\text{total tricarboxylic acids}}$$

shows great variations with the experimental conditions is not surprising, because this ratio is the resultant of the rates of at least six reactions (indicated by the arrows in scheme 3) each of which varies with the conditions. Variability of the ratio does not invalidate the conclusion that the results of the experiments in Table 10 support scheme 3.

Note on non-enzymic condensation reactions of oxaloacetate. Breusch (1941) noted that neutral solutions of oxaloacetate may contain substances which simulate citric acid in analytical methods based on the formation of pentabromoacetone, and that the quantities of interfering substances increased on addition of calcium salts. Our own experiments confirmed this observation, and furthermore showed that substances arise by non-enzymic reactions of oxaloacetate, which behave like *iso*citrate and *cis*aconitate, forming bromo compounds after incubation with aconitase resembling pentabromoacetone. All substances interfering with the determination of citrate and of *cis*aconitate plus *iso*citrate are however quantitatively removed by boiling the acidified solution before treatment with KBr and $KMnO_4$, an obligatory step in the citric acid determination.

Effect of $BaCl_2$ *on the yields of citrate*

Wieland & Rosenthal (1943) and Weinhouse *et al.* (1946) have found that the yields of citrate from a mixture of oxaloacetate and acetoacetate increase if $BaCl_2$ (about 0·01 M) is added to the medium. Data given in Table 11 confirm this finding. Wieland & Rosenthal offer no explanation for the cause of this effect. On the basis of the following scheme one would expect the yield of citrate to rise if a specific inhibitor reduced the rates of (*b*) or (*d*) without major effects on the rates of (*c*). In order to test whether barium ions inhibit specifically (*b*), aconitase (minced sheep heart muscle) was incubated with *cis*aconitate in the presence and absence of

Table 10. *Formation of citrate,* cis*aconitate and* iso*citrate in minced sheep kidney cortex*

(Tissue suspended in 9 parts medium. The data refer to 24 ml. suspension. The media did not contain Ca ions; pH in all cases 7·4.)

Exp. no.	Medium	Gas	Substrate added (final concentrations)	Period of incubation (min.)	Citrate formed (μl.)	*cis*Aconitate + *iso*citrate formed (μl.)	*cis*Aconitate + *iso*citrate as % of total tricarboxylic acids
I	Phosphate saline	O_2	0·01 M-Oxaloacetate	5	128	47	26·8
			0·01 M-Oxaloacetate	10	227	90	28·4
			0·01 M-Oxaloacetate, 0·005 M-acetoacetate	5	229	57	19·9
			0·01 M-Oxaloacetate, 0·005 M-acetoacetate	10	350	268	43·5
II	Phosphate-bicarbonate saline	5 % CO_2 in O_2	0·02 M-Oxaloacetate, 0·02 M-pyruvate	30	1600	1560	49·4
			0·02 M-Oxaloacetate, 0·02 M-pyruvate, 0·01 M-acetoacetate	30	3200	~ Nil	—
III	Phosphate saline	O_2	0·01 M-Oxaloacetate	10	190	22	10·4
			0·01 M-Oxaloacetate, 0·005 M-acetoacetate	10	415	47	10·2
IV	Phosphate-bicarbonate saline	5 % CO_2 in O_2	0·02 M-Oxaloacetate	20	316	~ Nil	—
			0·02 M-Oxaloacetate, 0·02 M-pyruvate	20	580	~Nil	—
			0·02 M-Oxaloacetate, 0·01 M-acetoacetate	20	1000	430	30·1
			0·02 M-Oxaloacetate, 0·01 M-acetoacetate, 0·02 M-pyruvate	20	1240	124	9·1

BaCl$_2$ and the rates of formation of *iso*citrate and citrate were measured.

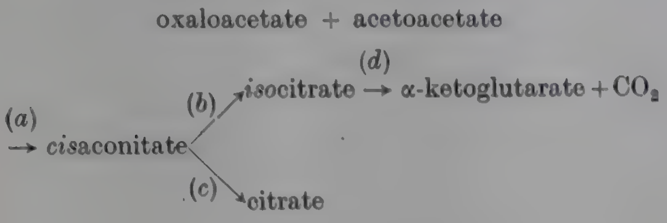

oxaloacetate + acetoacetate
(a)
→ *cis*aconitate
(b)
*iso*citrate → α-ketoglutarate + CO$_2$
(d)
(c)
citrate

Table 11. *Effects of* BaCl$_2$ *on yields of citrate from oxaloacetate and acetoacetate*

(Minced sheep kidney cortex suspended in 9 parts phosphate-bicarbonate saline. Period of incubation 30 min.; 5% CO$_2$ in O$_2$; 0·02 M-oxaloacetate; 0·01 M-acetoacetate. The data refer to 4 ml.)

	Without BaCl$_2$	With 0·02 M-BaCl$_2$
Oxaloacetate removed (μl.)	1310	1200
Acetoacetate reduced (μl.)	72	53
Acetoacetate 'oxidized' (μl.)	258	329
Citrate formed (μl.)	162	344
L-Malate formed (μl.)	189	169

Table 12 demonstrates the phenomenon discovered by Martius (1938) that during the early stages of incubation *iso*citrate is formed more rapidly than citrate, and that *iso*citrate is later reconverted to citrate. BaCl$_2$ (0·02 M) slows down both reactions (*b*) and (*c*) a little. At 10 min. the inhibition of (*b*) is 17%, of (*c*) 28%. Thus the increased yield of citrate from oxaloacetate and acetoacetate cannot be explained by a specific inhibition of reaction (*b*).

With and without barium chloride the maximum yields of *iso*citrate are 23% of the added *cis*aconitate, whilst the final yield is only 5%. Probably owing to the conversion of some *cis*aconitate into the *trans* form during the prolonged incubation the total yield of *cis*aconitate and *iso*citrate was less than that obtained with more powerful aconitase preparations in short-term experiments (Krebs & Eggleston, 1943).

As for effects of barium on reaction (*d*) (the oxidative removal of *iso*citrate), Table 13 indicates that barium ions inhibit the O$_2$ uptake of muscle without citrate (see Thunberg, 1937), but citrate increases the oxygen uptake also in the presence of Ba and the rate of removal of citrate is only slightly reduced by Ba. This effect is too small to account for the increased yields of citrate and by the method of elimination one is therefore led to the assumption that the rate of *cis*aconitate formation (reaction *a*) is accelerated in the presence of barium ions. Such an

Table 12. *Effect of* BaCl$_2$ *on aconitase*

(4·2 g. frozen minced sheep heart suspended in a total volume of 100 ml.; 0·025 M-phosphate buffer, pH 7·4; 0·1 M-*cis*aconitate = 10 mmol. 40°. At intervals, 10 ml. solutions were removed for analysis and the reaction was stopped by addition of 1·25 ml. glacial acetic acid. *iso*Citrate was determined polarimetrically in 9 ml., citrate in 0·5 ml. The quantities of *iso*citrate and citrate are expressed in mmol.)

	No BaCl$_2$ added		0·02 M-BaCl$_2$	
Time (min.)	*iso*Citrate (mmol.)	Citrate (mmol.)	*iso*Citrate (mmol.)	Citrate (mmol.)
0	0	0	0	0
5	1·17	—	1·03	—
10	1·82	1·70	1·52	1·23
20	2·26	—	1·93	—
30	2·21	3·39	2·17	2·02
40	1·93	—	2·28	—
60	1·42	4·65	2·23	3·12
90	0·91	—	1·94	—
120	0·67	6·61	1·72	4·58
180	0·53	6·58	1·41	5·24
24 hr.	0·56	6·79	0·51	6·80

Table 13. *Effect of* BaCl$_2$ *and the composition of saline medium on* O$_2$ *uptake and citrate removal in sheep kidney cortex*

(Minced sheep kidney suspended in 9 parts medium. 40°. 60 min. Medium I = phosphate saline, pH 7·4; medium II = 45 ml. 0·9% NaCl + 4·8 ml. M-NaHCO$_3$. (Breusch's (1943) medium). O$_2$.)

	Medium I				Medium II			
Substances added to medium	Nil	0·005 M-Citrate	0·01 M-BaCl$_2$	0·005 M-Citrate, 0·01 M-BaCl$_2$	Nil	0·005 M-Citrate	0·01 M-BaCl$_2$	0·005 M-Citrate. 0·01 M-BaCl$_2$
O$_2$ uptake (μl.)	1290	1790	830	1150	193	310	351	339
Citrate removed (μl.)	—	432	—	355	—	106	—	96

acceleration is feasible bearing in mind that a number of oxidative reactions in respiring cells compete with, and thus inhibit, each other; lactate, e.g., inhibits the oxidation of L-amino acids (see Krebs, 1935). It is feasible that barium ions inhibit a reaction which normally compete with, and thus slows down, the formation of citrate. This would result in an increased rate of citrate formation.

According to Breusch (1943*a*, *b*) the presence of bicarbonate in the medium, without CO_2 in the gas phase, also increases the yields of citrate from oxaloacetate. Table 13 shows that the oxygen uptake and citrate removal are greatly reduced in Breusch's medium. The increased yields of citrate in this case might thus be explained by the assumption that in this medium, which is unphysiologically alkaline, the formation of citrate is less inhibited than its removal.

DISCUSSION

Acetoacetate and the tricarboxylic acid cycle. The facts that the C_4-dicarboxylic acids promote the aerobic removal of acetoacetate and that acetoacetate increases the total yield of the acids of that cycle in the presence of oxaloacetate are in accordance with the conception, already safely established by tracer work, that acetoacetate is metabolized through the tricarboxylic cycle. So far there is no conclusive evidence suggesting alternative pathways of acetoacetate breakdown.

The main gap in the present knowledge of acetoacetate metabolism concerns the reactions leading from acetoacetate and oxaloacetate to *cis*aconitate. It is noteworthy that these reactions appear to require the presence of O_2, although the balance sheet of the reaction

$$\text{acetoacetate} + \text{oxaloacetate} \rightarrow cis\text{aconitate}$$

does not represent an oxidation.

Our previous arguments (Krebs & Eggleston, 1945) against the hypothesis of Breusch (1943*a*, *b*) and of Wieland & Rosenthal (1943) were based on anaerobic experiments, where practically all the acetoacetate removed in the presence of oxaloacetate was accounted for as β-hydroxybutyrate. We pointed out that Breusch and Wieland & Rosenthal had supplied no evidence that under their experimental conditions acetoacetate was removed by a reaction other than reduction and that an alternative explanation was available for the increased yields of citrate. These arguments are now superseded by the tracer work of Buchanan *et al.* (1945) and Weinhouse *et al.* (1945, 1946).

Mechanism of condensation. The following considerations are offered as a contribution to the unsolved problem of the mechanism whereby oxaloacetate condenses with a second molecule to form the 6-carbon skeleton of the tricarboxylic acids. The tracer experiments have made it clear that citrate cannot be an intermediate because the isotopic carbon introduced as $CH_3.^{13}CO.CH_2.^{13}COOH$ was unsymmetrically distributed over the two carboxyl groups of α-ketoglutarate, the major part being in the γ-carboxyl. These facts are in accordance with the view that *cis*aconitate is the primary product of condensation provided that the reaction *cis*aconitate $\rightarrow$ *iso*citrate $\rightarrow$ α-ketoglutarate is very rapid in comparison with the reaction *cis*aconitate $\rightarrow$ citrate. Experimental evidence supporting this assumption has been presented previously (Krebs, 1943).

By analogy with well known organic chemical reactions a possible mechanism of formation of *cis*aconitate would be an aldol condensation of the type of the Perkin reaction or Knoevenagel reaction:

$$R.CHO + CH_3COONa \rightarrow R.CH{:}CH.COONa.$$

Applied to oxaloacetate and an acetate residue this type of condensation would yield a *cis*aconitic acid with the double bond in the 2:3 position to the carboxyl of the acetate residue. This would form an α-ketoglutarate whose carboxyl adjacent to the α-ketonic group is derived from the carboxyl of the acetate residue. The tracer experiments show that this is not the case. No double bond is formed as a result of the enzymic condensation. The double bond in *cis*aconitic acid, as already assumed by Wood *et al.* (1941), must be derived from that of the *enol*oxaloacetate (hydroxymaleic acid).

SUMMARY

1. The aerobic removal of acetoacetate by minced sheep heart and kidney cortex was investigated and a number of metabolites formed in the presence of acetoacetate were determined.

2. Manometric methods were developed for the determination of pyruvate in the presence of oxaloacetate and of oxaloacetate in the presence of pyruvate plus acetoacetate. In elaborating these methods it was noted that oxaloacetate gives variable yields of CO_2 with yeast carboxylase and that mixtures of acetoacetate and α-ketonic acids (pyruvate, α-ketoglutarate) behave towards Al ions like dicarboxylic β-ketonic acids, which suggests that these mixtures contain β-ketonic acids formed by aldol condensation.

3. In heart and kidney cortex some of the added acetoacetate was rapidly reduced to β-hydroxybutyrate even under aerobic conditions, whilst another fraction underwent oxidation. The rate of oxidation was increased by oxaloacetate and by fumarate. Data on the rates of reduction and oxidation of acetoacetate under varying conditions are given.

4. Nine metabolites (oxaloacetate, acetoacetate, pyruvate, β-hydroxybutyrate, succinate, fumarate,

L-malate, citrate, α-ketoglutarate) found in solutions as a result of the interaction between acetoacetate and oxaloacetate were determined in aerobic and anaerobic experiments. The most notable effect of oxygen on the yields of various substances was a higher yield of citrate. Calculations based on the yields suggest that acetoacetate contributes towards the formation of the acids of the tricarboxylic cycle, a conclusion now firmly established by the tracer work of Buchanan *et al.* and of Weinhouse *et al.*

5. Erythro-1:2-dihydroxybutyric acid was not metabolized by sheep kidney cortex whilst the threo form was slowly oxidized. In accordance with Weil-Malherbe's findings, the slowness of the oxidation excludes this substance as an intermediate in the removal of ketone bodies.

6. Measurements of the rates of α-ketoglutarate formation from various substrates and substrate combinations showed that the tricarboxylic acids yield α-ketoglutarate more rapidly than any other substrate. This supports the assumption that α-ketoglutarate arises via the tricarboxylic acids when formed in the presence of oxaloacetate and acetoacetate (or pyruvate). The rate of citrate formation via carboxylation of α-ketoglutarate (Ochoa, 1945) was relatively slow in sheep kidney cortex.

7. The sum of *cis*aconitate plus *iso*citrate was found to exceed 10·5 % of the total tricarboxylic acids (maximum 49 %) under suitable conditions. It is pointed out that this finding excludes the sequence (4) (p. 302) and supports the sequence (3) (p. 301), confirming conclusions earlier drawn from tracer work.

8. It was confirmed that barium (Wieland & Rosenthal, 1943) and bicarbonate (Breusch, 1943*a*, *b*) ions increase the yield of citrate from oxaloacetate and acetoacetate. Possible causes of this effect were investigated.

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The Isolation of Hydroxylysine Picrate from a Gelatin Hydrolysate

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Schryver, Buston & Mukherjee (1925) claimed the isolation, from isinglass hydrolysate, of an 'oxylysine' to which they attributed the formula $C_6H_{14}O_3N_2$; but they were unable, on repeated attempts, to prepare a crystalline picrate. Van Slyke & Hiller (1921) reported the presence of hitherto unknown material in the basic amino-acid fraction of hydrolyzed gelatin; this base was isolated by Van Slyke, Hiller, Dillon & MacFadyen (1938) who ascribed to it the formula of a hydroxylysine. After removal of arginine and histidine, these workers freed the 'lysine fraction' from monoamino-acids, and then removed the greater portion of the lysine by the addition of sufficient picric acid to combine with three quarters of the amino nitrogen present. A picrate of composition $C_6H_{14}O_3N_2 . C_6H_3O_7N_3$, which melted at 225° when heated slowly, was eventually isolated from the mother liquor. Van Slyke, Hiller, MacFadyen, Hastings & Klemperer (1940) and Van Slyke, Hiller & MacFadyen (1941) have since endeavoured to establish the structure of hydroxylysine, and to determine its presence in other proteins by adaptation of the periodate oxidation reaction of Nicolet & Shinn (1939).

Martin & Synge (1941), by the application of an acetylation-benzoylation procedure to the lysine fractions of gelatin and isinglass hydrolysates, isolated in each case a small amount of a crystalline picrate which exploded at 226–227°. Although their analytical figures for formaldehyde and ammonia evolved on periodate treatment were low, this work is the only independent confirmation, by isolation, of the existence of the base first described by Van Slyke *et al.* (1938).

Additional evidence has been forthcoming from the periodate reaction besides that supplied by Martin & Synge. Using Vorländer's (1929) technique for determining the formaldehyde produced by oxidation of the 'lysine fraction' of isinglass, Beveridge & Lucas (1944) obtained a value from which they deduced the probable presence of some 0·36 % hydroxylysine. It was not apparent whether the compound estimated was that described by Schryver *et al.* (1925), or by Van Slyke *et al.* (1938), and accordingly they attempted the isolation advocated by the American workers, but failed to obtain any hydroxylysine picrate. More recently, Rees (1946) has carried out estimations by periodate oxidation which have confirmed the presence of a basic hydroxy amino-acid in gelatin and collagen, but not in any other proteins examined. Desnuelle & Antonin (1947) have criticized Van Slyke's figures based on the production of ammonia in the periodate reaction when carried out in the presence of excess glycine, and have preferred a colorimetric estimation of the formaldehyde simultaneously liberated. They found no evidence for the presence of hydroxylysine in casein, which contains, according to Van Slyke and his co-workers, 0·33 %. Neither Rees (1946) nor Desnuelle & Antonin (1947) attempted the isolation of any derivatives.

The isolation of a hydroxylysine picrate, which agrees in melting point and other physical properties with the compound isolated by Van Slyke and which affords formaldehyde and ammonia on periodate oxidation is described in this paper. The separation was effected by means of fractional recrystallization of the product derived from the 'lysine fraction' of a purified gelatin catholyte.

MATERIALS AND METHODS

Gelatin (1 kg.) known commercially as 'Spa Brand', kindly supplied by Messrs B. Young and Co. Ltd., of Bermondsey and containing 2 % ash, 15 % moisture and 13·82 % (uncorr.) nitrogen was hydrolyzed by boiling for 24 hr. with 2·5 l. of 30 % (v/v) H_2SO_4; after dilution, the H_2SO_4 was removed first to pH 2·5 and finally to pH 5·5 with $Ba(OH)_2.8H_2O$. The hydrolysate, together with washings of the precipitates, was then concentrated *in vacuo* to *c.* 1 l., and subjected to fractional electrodialysis in a three-compartment cell to remove all but traces of monoamino-acids from the catholyte. From this purified basic fraction, arginine and histidine were completely removed with flavianic acid, and excess flavianic acid with baryta. Finally the excess Ba^{++} was entirely removed with H_2SO_4 and ammonia by concentration *in vacuo*.

EXPERIMENTAL

Main picrate fraction

Preparation. The 'lysine fraction' referred to above (700 ml.) was precipitated with 45 g. (excess) of picric acid, affording a picrate which, after recrystallization from 1 l. of water, weighed 24 g. and exploded at 267°, the usually accepted explosion point for lysine picrate. Recrystallization of this picrate from water produced no change in explosion point.

Conversion to dihydrochloride. A portion (10 g.) of this twice recrystallized main picrate fraction was converted into the dihydrochloride (Cox, King & Berg, 1929). Although free from arginine, histidine and ammonia, the product melted at 192–193°, i.e. about 10° below the accepted melting point of lysine dihydrochloride and was also very hygroscopic.

Analysis of the dihydrochloride by HIO_4 *oxidation.* The dihydrochloride (0·1 g.), prepared as above, was dissolved in water (5 ml.) and HIO_4 (0·1 g. in 10 ml. of water) added. The oxidation was carried out in the manner described by Van Slyke *et al.* (1940). Subsequent treatment with Vorländer's reagent, 'dimedone' (5:5-dimethyl*cyclo*hexane-1:3-dione) precipitated an insoluble condensation product which was identified by its m.p. (185°) as methylene bis-dimedone. The melting point of this compound was not depressed on admixture with authentic material (m.p. 189°). Assuming the formaldehyde to have been derived from a hydroxylysine, the yield of dimedone derivative, 1·61 mg., corresponds to the presence of some 1·3 g. of the hydroxylysine salt/100 g. of dihydrochloride. (This figure must be regarded as minimal and the true content may well be higher.)

The suggestive analytical evidence obtained from the main picrate fraction warranted a search for greater quantities of the presumptive hydroxylysine in the main picrate mother liquors. Fractionation of these did, indeed, lead to the isolation of a picrate having the properties of that of hydroxylysine.

Fractionation of the main picrate mother liquors

The mother liquor and the washings (*c.* 1 l.) from the main crop of picrate were evaporated to 50 ml. affording, after one recrystallization, a second crop P_1 (1·9 g.) of explosion point 254°. Recrystallization of P_1 from water yielded 1·76 g. of P_2 (explosion point 259°). From the filtrate and washings, containing 0·14 g. of mixed picrates, was obtained, after evaporation to a few ml. and cooling to room temperature, a picrate P_3, paler yellow in colour than the picrate of lysine.

P_3, after filtration, washing with ice water and drying *in vacuo*, melted at *c.* 234°, with decomposition and bubbling but without explosion. Recrystallization from the minimum amount of water gave picrate P_4 of similar melting point and so the solvent was changed to 50 % (v/v) aqueous ethanol. This solution was boiled with charcoal, filtered and evaporated to dryness to give P_5. The latter was taken up in boiling methanol containing a little water and allowed to crystallize at 5°. The picrate so formed melted with decomposition at 225°, and this melting point was unchanged on further recrystallization from aqueous ethanol.

Properties of the picrate isolated

The compound was finally isolated in a yield of some 80 mg. When heated at the rate of 1° every 3 sec., it showed the same m.p. (225°) as the hydroxylysine picrate isolated by Van Slyke *et al.* (1938). The change was fairly sharp, but, though decomposition took place at the same time, there was no explosion such as occurs with lysine picrate. The intermediate fractions, P_3, P_4, showed the typical decomposition of impure lysine picrate. The crystals of the new compound, which consisted of pale-yellow needles possessing a tendency to sheaf-like formation, appeared under the microscope very similar in shape to those of lysine picrate. Crystallization in the test tube took place rapidly from a supersaturated solution and a fluffy precipitate then formed. This dull, lemon-yellow precipitate was quite different in appearance from the characteristic, bright, golden-yellow colour associated with the picrate of lysine.

Oxidation by periodate. Samples of about 20 mg. each of the new picrate were treated with $0{\cdot}5\,\mathrm{M}\text{-}\mathrm{HIO}_4$, and the resulting formaldehyde was precipitated as the dimedone derivative, washed and dried at 110° to constant weight. Duplicates agreed to within 2·4%, but the yield of methylene bis-dimedone varied with the conditions of condensation. It is now known, from the work of MacFadyen (1945), in which the dimedone-formaldehyde condensation was studied by means of the chromotropic acid reaction that, for 'complete' (98%) precipitation, up to 29 hr. at 39°, may be necessary if the condensation is carried out at pH 3·5, though the time required rapidly diminishes as the pH rises. At the time that these experiments were being carried out, however, it was not realized that the conditions of precipitation were so important. In all experiments, precipitation was carried out in a medium which was acid to methyl red and at room temperature. In one set of experiments, where the mixture was cooled immediately after the addition of dimedone and then allowed to stand in the refrigerator for 18 hr., the yield corresponded to only 62% of theory for hydroxylysine picrate; when the mixture was allowed to stand at room temperature for about 3 hr. before refrigeration, the yield rose to 81·8%.

Lack of material prevented the carrying out of further analyses. There seems little doubt, however, when the results of the periodate oxidation are considered in conjunction with the other properties of the picrate isolated from these mother liquors, that the base in question was hydroxylysine. The claims of Van Slyke *et al.* (1938) are thus confirmed.

DISCUSSION

It appeared impossible to purify the picrate mixture P_3, melting at *c.* 234°, by further recrystallization from water although, on changing the solvent, the heterogeneity of the substance became apparent and eventually hydroxylysine picrate of lower melting point was separated from the mixture. Thus the evidence strongly indicates that a solid solution separates from the aqueous system.

In this connexion, it may be pointed out that Van Slyke *et al.* (1941) found that diamino-acids are precipitated from mixtures by phosphotungstic acid partly as mixed salts rather than as individual substances each with its own independent solubility. These authors have shown further that in a mixture of lysine and hydroxylysine so precipitated the amount of hydroxylysine in the filtrate varies inversely to the ratio of lysine/hydroxylysine in the precipitate. The observations contained in this paper on the picrates are in good agreement with the above, and are of some practical importance in indicating that the isolation of hydroxylysine, whether by fractional precipitation with picric acid or by fractional recrystallization, depends ultimately on the solubilities of the picrates in water and in one another.

In view of this evidence, it would seem unlikely that hydroxylysine of 100% purity could be obtained by ordinary purification procedures. Gale (1947) has recently tested a decarboxylase, claimed to be specific for L-lysine, on samples of hydroxylysine received from Van Slyke. These samples were slowly, but incompletely, attacked. Zittle & Eldred (1944), using the same organism on zein hydrolysates, have shown that hydroxylysine cannot be decarboxylated to any appreciable extent. Although the amounts of lysine and hydroxylysine in zein are somewhat uncertain, it is possible that the results of Gale (1947) are due, in part, to contamination of the hydroxylysine with lysine.

SUMMARY

1. Hydroxylysine, the amino-acid prepared from gelatin by Van Slyke and co-workers has been independently isolated by fractional recrystallization of its picrate, from a sample of crude lysine picrate. Some of the properties of hydroxylysine picrate have been described.

2. The difficulties encountered in the separation of hydroxylysine picrate from that of lysine by fractional crystallization are stressed, and evidence is produced indicating that even the best samples of hydroxylysine so far prepared are not entirely free of lysine.

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The Reaction of Nucleic Acids with Mustard Gas

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The delayed healing of lesions caused by mustard gas (H) provoked speculation that the cell nuclei are especially concerned, and confirmation of this view has accumulated from various sources. Auerbach & Robson (1942, 1946) showed that the exposure of *Drosophila melanogaster* to H in sublethal doses caused reduced fertility and induced visible mutations in succeeding generations. Robson (1943) observed that pollen grains of *Tradescantia* after treatment with H, showed widespread disorganization of chromosomes, and Fell & Allsopp (1943) noted similar behaviour in the cells of H-treated mouse skin. Koller (1943) records that during the 48 hr. following a short (30 sec.) exposure of *Tradescantia* pollen grains to concentrated H vapour, chromosome breakage, structural changes including new rearrangements, and adherences of adjacent chromosomes were induced at various loci in the chromosomes. He observed that the separation of the chromosomes at anaphase and telophase of mitosis was prevented by these attachments. These results focused attention on the action of H on nucleoproteins, and Berenblum (1940 *a*, *b*) and Berenblum & Schoental (1947) found that aqueous extracts of skin or solutions of thymus nucleoprotein underwent irreversible precipitation when treated with H, the precipitate having a higher sulphur content than the original nucleoprotein.

The action of H on proteins has been studied by many investigators, e.g. Berenblum & Wormall (1939); for reviews see Rydon (1943 *a*, *b*). The purpose of the present investigation was therefore to investigate the reaction of H with nucleic acids and their components.

EXPERIMENTAL

Choice of nucleic acids for investigation. The ribose nucleic acid of yeast (YNA) and the deoxypentose nucleic acid of calf thymus gland (TNA) were employed in this investigation because of their availability, and because more is known of their structures than of those of nucleic acids from other sources.

Preparation of materials. The samples of yeast ribonucleic acid were obtained commercially and were deproteinized by a combination of the method described by Sevag, Lackmann & Smolens (1938) and the purification process employed by Fletcher, Gulland, Jordan & Dibben (1944). Yeast ribonucleic acid (20 g.), stirred vigorously into water (250 ml.), was brought into solution at pH 7 by the addition of N-NaOH (about 48 ml.). This solution was shaken vigorously with the following volumes of a mixture (4 : 1) of chloroform and amyl alcohol: 200 ml. four times, 100 ml. ten times. After each shaking, the mixture was centrifuged and the aqueous layer containing the nucleic acid was separated from the chloroform layer which contained the protein as a stable gel. When gel formation no longer occurred after shaking, the solution of protein-free nucleic acid was slowly added to a stirred mixture of ethanol (1180 ml.) and concentrated HCl (6·6 ml.). The precipitated acid (12 g.) was centrifuged and washed three times with 66% aqueous ethanol when it was found to be free from chloride ions; it was then washed successively, once with 80 and 100% ethanol, finally with ether, and dried in a vacuum over P_2O_5-KOH at room temperature. Analyses of various samples are recorded in Table 2.

Deaminated yeast ribonucleic acid (DYNA) was prepared from YNA as described by Fletcher, Gulland, Jordan & Dibben (1944). The samples of the sodium salt of deoxypentose nucleic acid of low molecular weight were obtained commercially and deproteinized by the procedure described above. The sample of high molecular weight had been prepared from calf thymus glands by Gulland, Jordan & Threlfall (1947). Analyses are given in Table 2. Guanylic acid in the form of its sodium salt was prepared by alkaline hydrolysis of YNA. The pure *H* used was provided by the Chemical Defence Research Department, Porton.

Table 1. *Conditions of reaction of H with nucleic acids*

Reference no. of nucleic acid	Wt. of nucleic acid (g.)	Wt. of *H* (g.)	Mol. of *H*/tetra-nucleotide	Vol. of solution (ml.)	Time of stirring (hr.)	Reference no. of A product	Yield of A product (g.)	Reference no. of B product	Yield of B product (g.)	Yield of A / Yield of B
YNA:										
Y 3/20	1·50	0·75	4·1	8·0	10·0	HY 3/22 A	1·00	HY 3/22 B	0·55	1·82
Y 4/26	4·00	5·00	10·2	30·0	6·0	HY 4/30 A	3·60	HY 4/30 B	0·30	12·00
Y 5/42	3·00	1·90	5·1	20·0	10·0	HY 5/45 A	1·40	HY 5/45 B	0·90	1·56
Y 5/42	3·00	1·90	5·1	40·0	10·0	HY 6/46 A	1·15	HY 6/46 B	0·85	1·35
Y 5/42	3·00	1·90	5·1	60·0	10·0	HY 7/47 A	1·50	HY 7/47 B	0·75	2·00
Y 6/48	3·00	1·90	5·1	20·0	3·0	HY 8/49 A	0·10	HY 8/49 B	3·05	0·03
Y 6/48	3·00	1·90	5·1	20·0	6·0	HY 9/50 A	0·50	HY 9/50 B	2·55	0·20
DYNA:										
DY 2	0·50	0·25	4·1	8·0	22·0	HDY 1/25 A	0·15	HDY 1/25 B	0·25	0·60
DY 8	6·80	8·00	9·6	48·0	7·0	HDY 2/34 A	2·18	HDY 2/34 B	4·10	0·53
HDY 2/34 B	2·00	3·00	13·7	20·0	10·0	HHDY 3/43 A	1·00	HHDY 3/43 B	0·10	10·00
TNA:										
T 1/19*	0·75	0·70	7·3	8·0	10·0	HT 1/27 A	0·75	HT 1/27 B	0·02	37·5
T 2/31*	1·50	1·50	7·3	23·0	11·0	HT 2/36 A	1·15	HT 2/36 B	0·35	3·3
T 5†	2·00	1·30	5·8	450·0	9·0	HT 3/53 A	0·01	HT 3/53 B	1·60	0·006

YNA = yeast nucleic acid; DYNA = deaminated yeast nucleic acid; TNA = thymonucleic acid.
* Degraded low mol. wt. samples. † High mol. wt. sample (tetrasodium salt).

Table 2. *Analytical data for H-treated nucleic acids*

Nucleic acid			A product					B product			
Reference no.	N (%)	P (%)	Reference no.	S (%)	N (%)	P (%)	Na (%)	Reference no.	S (%)	N (%)	P (%)
YNA:											
Y 3/20	16·7	9·1	HY 3/22 A	4·4	14·3	7·6	—	HY 3/22 B	4·3	14·9	8·2
Y 4/26	16·5	9·4	HY 4/30 A	3·1	14·5	8·1	3·2	HY 4/30 B	2·8	14·4	8·6
Y 5/42	15·9	9·2	HY 5/45 A	3·5	13·6	7·7	—	HY 5/45 B	3·8	13·5	8·3
Y 5/42	15·9	9·2	HY 6/46 A	4·3	13·2	7·5	—	HY 6/46 B	4·7	13·5	8·2
Y 5/42	15·9	9·2	HY 7/47 A	4·9	13·4	7·6	—	HY 7/47 B	5·2	13·3	7·9
Y 6/48	15·6	9·2	HY 8/49 A	1·6	14·0	7·7	—	HY 8/49 B	1·5	15·0	8·8
Y 6/48	15·6	9·2	HY 9/50 A	3·0	14·0	7·6	—	HY 9/50 B	2·8	14·7	8·4
DYNA:											
DY 2	11·7	7·6	HDY 1/25 A	5·0	—	—	—	HDY 1/25 B	5·1	11·2	7·4
DY 8	—	—	HDY 2/34 A	4·3	10·6	7·2	3·4	HDY 2/34 B	4·6	10·4	7·9
HDY 2/34 B	10·4	7·9	HHDY 3/43 A	7·9	9·2	6·7	—	HHDY 3/43 B	6·7	8·3	7·5
TNA:											
T 1/19*	—	—	HT 1/27 A	3·4	13·9	8·6	1·0	HT 1/27 B	—	—	—
T 2/31*	16·4	9·5	HT 2/36 A	3·7	13·3	8·2	3·4	HT 2/36 B	3·5	12·9	8·9
T 5†	15·3	9·3	HT 3/53 A	—	—	—	—	HT 3/53 B	4·9	13·3	8·6

YNA = yeast nucleic acid; DYNA = deaminated yeast nucleic acid; TNA = thymonucleic acid.
* Degraded low mol. wt. samples. † High mol. wt. sample (tetrasodium salt).

Criteria of reaction of H with nucleic acids. The criteria of positive chemical reaction between *H* and nucleic acids were as follows: (i) the qualitative demonstration of the presence of sulphur in the products, followed later by detailed analytical investigation; (ii) the demonstration of the absence of uncombined *H* in the products by prolonged passage of air from the sample through S.D. (1942) reagent paper. (Before these tests were made, the samples were thoroughly washed with ethanol and ether.) The absence of free *H* in the products was confirmed by the consistent failure to detect the presence of chlorine as a component by means of qualitative tests.

The reaction of H with nucleic acids. No interaction could be detected between YNA and *H* as vapour or dissolved in liquid paraffin. When, however, both were dissolved in aqueous ethanol at pH 7, a reaction occurred. The solubility of *H* in aqueous ethanol increases with the concentration of alcohol, whereas that of nucleic acids decreases. The highest concentration of ethanol which could be employed without the precipitation of nucleic acid was 35%, and this concentration was employed throughout the experiments described below; the presence of ethanol, moreover, considerably reduces the hydrolysis of *H* (Mohler & Hartnagel, 1941; Ogston, 1941) which is a serious competing reaction. When the reaction was carried out in aqueous media with *H* in suspension, the reaction between *H* and nucleic acid was found to be almost negligible.

The results of a series of experiments made with different proportions of *H* and YNA are summarized in Tables 1 and 2. Two products, designated A and B respectively, were always formed, but in various proportions depending on the conditions of the experiment. The A compounds separated from the reaction mixture at pH 7 during the course of the experiment, whereas the B compounds were precipitated by the addition of a mixture of HCl and ethanol after removal of the A compounds at the conclusion of the preparation.

The following description is typical of the experiments which were carried out, and illustrates the procedure. Yeast ribonucleic acid (Y 5/42; 3 g.) was suspended in water and brought into solution at pH 7 by the addition of approximately N-NaOH (about 7 ml.), the final volume being 13 ml. Ethanol (7 ml.) was added, then *H* (1·9 g.), and the mixture was stirred mechanically for 10 hr., the reaction being frequently adjusted to pH 7 by the addition of NaOH. The solid, a sodium salt which had separated, was centrifuged, washed several times with dry ethanol, twice with ether, and dried *in vacuo* over P_2O_5-KOH. Yield 1·4 g. (HY 5/45 A).

The supernatant liquid was then added slowly to a stirred mixture of ethanol (110 ml.) and concentrated HCl (0·6 ml.). The precipitated solid was centrifuged, washed with ethanol and then with ether, and dried *in vacuo* as before. Yield 0·95 g. (HY 5/45 B).

The reactions of *H* with DYNA and with TNA were carried out by a similar procedure. The results are summarized in Tables 1 and 2.

The reaction of H with guanylic acid. The method described above for the reaction of *H* with nucleic acids gave only poor yields when applied to the reaction between *H* and guanylic acid and the following modified procedure, which gave a satisfactory yield, was developed. Sodium guanylate (3 g.) was dissolved in hot water (30 ml.), the solution cooled and added to a solution of *H* (11·8 g., 10 mol. ratio) in ether (15 ml.). The mixture was stirred for 72 hr. and kept neutral by periodic additions of NaOH. *H*-guanylic acid was precipitated with ethanol-ether (5 : 1) mixture (450 ml.). The gummy product was centrifuged and washed four times with ethanol and twice with ether. It was redissolved in water (50 ml.) and the *H*-guanylic acid reprecipitated as a solid by the addition of ethanol and ether; a further reprecipitation was necessary to free the product from NaCl. The final separation of the product by centrifuging was exceedingly difficult owing to its colloidal nature. The *H*-guanylic acid (2·1 g.) was dried *in vacuo* over P_2O_5.

Analytical methods. Nitrogen analyses of untreated and *H*-treated materials were carried out by a micro-Kjeldahl technique (Claudatus, 1939) modified by Still (unpublished). Phosphorus contents were determined by a modification (Still, unpublished) of the micro-method of Embden (1921).

Estimations of the sulphur content of *H*-nucleic acids were carried out by the adaptation by Niederl & Niederl (1938) of the Pregl combustion method. The accuracy of this method depends upon the presence of insufficient metal (e.g. sodium) to retain the S present as metallic sulphate. The *H*-nucleic acids conform to this requirement since the P : S ratio is relatively high. After the analysis, the phosphorus remained in the boat as sodium metaphosphate, which was in each case shown qualitatively to be free from sulphate. In some cases, estimations of sulphur were also made by the micro-Carius method; these results confirmed those obtained by the more rapid Pregl method.

The results of analysis for sulphur in *H*-guanylic acid were low and inconsistent by both the Pregl and Carius methods. In the Pregl method, sulphate could be detected in the boat, and the procedure was modified. The residue was extracted with very dilute HCl, the solution filtered and added to the main bulk of sulphate-containing solution; estimations of this sulphate as $BaSO_4$ then gave consistent results.

Sodium analyses were at first carried out by the method of Hoffmann & Osgood (1938). Later, however, a gravimetric method was used in which the sample was wet-ashed with concentrated HNO_3, phosphate removed as barium phosphate, and sodium weighed as Na_2SO_4.

Electrometric titrations were carried out by the method used by Fletcher, Gulland & Jordan (1944), corrections being applied as described by Jordan & Taylor (1946).

Viscosity measurements on *H*-TNA were carried out using Frampton's (1939) modification of the Ostwald viscometer.

RESULTS

Properties of H compounds

The A products from YNA were pale buff, amorphous solids, insoluble in water but dissolving near pH 10. The B products from YNA were cream-coloured solids, soluble in water above pH 3–4, but precipitated in more acid solutions.

The A compounds from DYNA were very similar in behaviour to those from YNA, being insoluble at a pH below 10. The B compounds were soluble in water and were not precipitated at pH 2·5; this solubility in acids is characteristic of DYNA (Fletcher, Gulland, Jordan & Dibben, 1944).

The A and B compounds from TNA resembled their analogues prepared from YNA in most respects, but exhibited at least one important difference in that their solutions were more viscous than those of the original TNA at corresponding concentrations. Aqueous solutions at pH 11–12 of the A compounds prepared from the low molecular weight samples T 1/19 and T 2/31 were far more viscous than the original nucleic acids, but quantitative determinations were not carried out. The viscosities of aqueous solutions of the sample T 5 of high molecular weight and of the B compound (HT 3/53 B) prepared from it are given for various pressures in Fig. 1. Solutions of HT 3/53 B exhibited critical pressures, in magnitude largely dependent on concentration; at pressures below the critical, the solutions formed a gel and did not flow. Critical pressures have not been determined for T 5 (Creeth, Gulland & Jordan, 1947), but if they exist are considerably lower than those of the B compound.

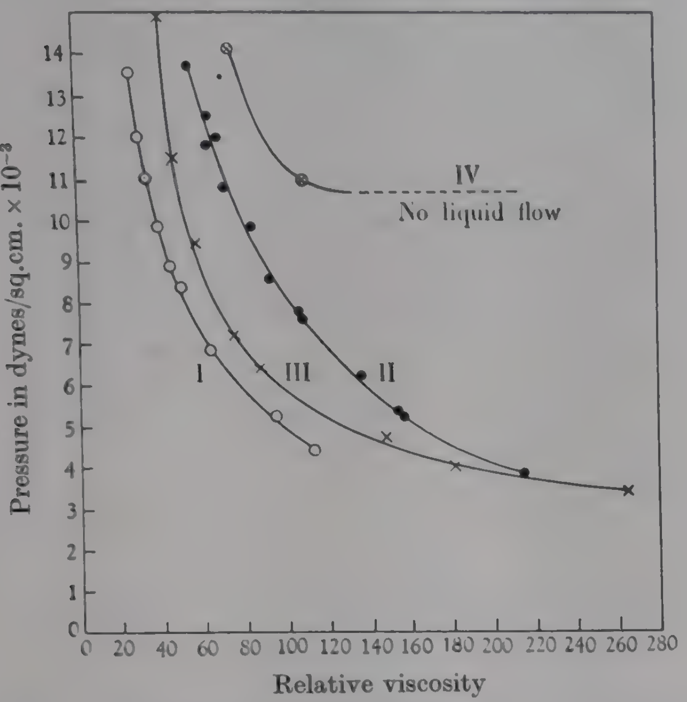

Fig. 1. The viscosities of aqueous solutions of calf thymus deoxypentose nucleic acid (T5) and its *H* product (HT3/53B). T5: curve I, ⊙, 0·21 % solution; curve II, ●, 0·31 % solution. HT3/53B: curve III, ×, 0·21 % solution; curve IV, ⊗, 0·31 % solution.

In air, these *H* compounds emitted a peculiar odour which was notably increased on treatment with acid and alkali. This odour was reminiscent of the thio-ethers and very similar to that obtained by the reaction of *H* with ethanolic alkali, known to give divinyl sulphide. In order to investigate the degree of the decomposition of the *H* compounds caused by the presumed evolution of divinyl sulphide, one sample was analyzed for sulphur immediately after preparation and again after storage for 5 weeks in a loosely corked tube at room temperature. The sulphur contents were 4·35 and 4·30 %, respectively. The difference between the two figures is within experimental error and hence no extensive decomposition had occurred.

The identification of groups reacting with H

The electrometric titration of nucleic acids has proved to be a useful method of investigating the structures of nucleic acids, and in particular the number and type of acidic and basic groups present (Fletcher, Gulland & Jordan, 1944; Gulland, Jordan & Taylor, 1947). The titration curves of the *H*-treated materials have therefore been determined to compare the number of titratable groups present with that of the original nucleic acids. In using this method for the *H*-nucleic acids, however, it is appreciated that titration may produce fission of the *H* residues from the nucleic acids, especially in strongly acid and alkaline solutions, and the results obtained may therefore be slightly in error. Throughout, the calculations of numbers of reacting groups are based for convenience on the provisional assumption, sufficiently accurate for present purposes, that the nucleic acids are composed of statistical tetranucleotides (Gulland, Barker & Jordan, 1945; Gulland, 1947).

In order to interpret the data obtained by elementary analysis and by electrometric titration of *H* compounds, it is necessary to estimate for each sample the formula weight (M) of a statistical *H* tetranucleotide. This was obtained in each case from the sulphur content, assuming that (i) the number of P atoms/statistical tetranucleotide was the same as in the parent substance, and (ii) the reaction consisted in the replacement of hydrogen atoms by $—CH_2.CH_2.S.CH_2.CH_2.OH$ or $(—CH_2.CH_2)_2S$ groups, the formula weight thus increasing by 104 in the first and 86 in the second case for each sulphur atom introduced.

In the case of substitution by

$$—CH_2.CH_2.S.CH_2.CH_2.OH,$$

if x such residues combine with each statistical tetranucleotide of the parent substance having formula weight m, and if the sulphur content of the product be s % then $s=3200x/(m+104x)$ and hence $x=sm/(3200-104s)$ and $M=m+104x$. Similarly, in the case of substitution by $(—CH_2.CH_2)_2S$ groups, $x=sm/(3200-86s)$ and $M=m+86x$.

The calculation of m presents no difficulty in the case of thymus deoxyribonucleic acid for which it has the value 1235, but in the case of yeast ribonucleic acid and its deamination product, allowance must be made for the deficiency in phosphorus below the theoretical value. Thus, if the percentage of phosphorus be p, m is now the formula weight of the hypothetical tetranucleotide corrected for the deficiency of phosphorus, and δ the atom deficiency of phosphorus/tetranucleotide, then the number of atoms of phosphorus/tetranucleotide is $4-\delta=mp/3100$. Since the loss of a phosphoryl group involves a decrease of 80 in the formula weight of the hypothetical tetranucleotide then $m=1285-80\delta$, and combining with the above equation $m=299{,}150/(310-8p)$.

The calculation of M for all *H* compounds of nucleic acid is thus possible and, using an analogous method, the formula weight of the *H* compound of guanylic acid can also be obtained.

The A products of *H*-TNA, *H*-YNA and *H*-DYNA were not sufficiently soluble in aqueous solutions to permit their examination by electrometric titration. The B products, however, were readily soluble, and the results of titrations performed with these compounds and *H*-guanylic acid are given in Figs. 2, 3, 4 and 5, together with the titration curves of the original substances. It was found to be immaterial whether glass or hydrogen electrodes were used, since poisoning of the latter did not occur. Table 3 summarizes the number of titratable groups potentially capable of reacting with *H*, the number of such groups remaining unsubstituted after treatment with *H*, and (by difference) the number of groups blocked by *H*. It will be seen that in every case a reaction with *H* has occurred.

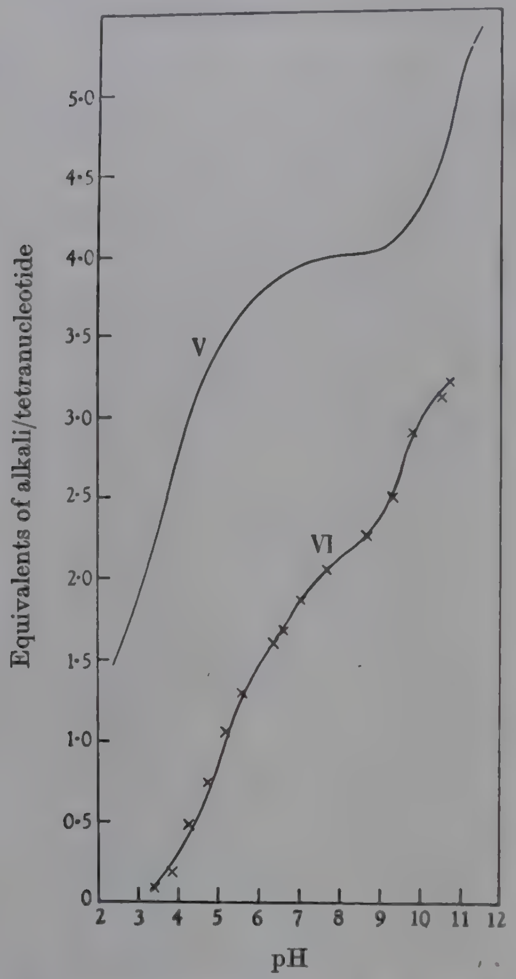

Fig. 2. The electrometric titration of calf thymus deoxypentose nucleic acid (T5), curve V (Gulland, Jordan & Taylor, 1947) and its *H* product (HT3/53 B), curve VI, ×.

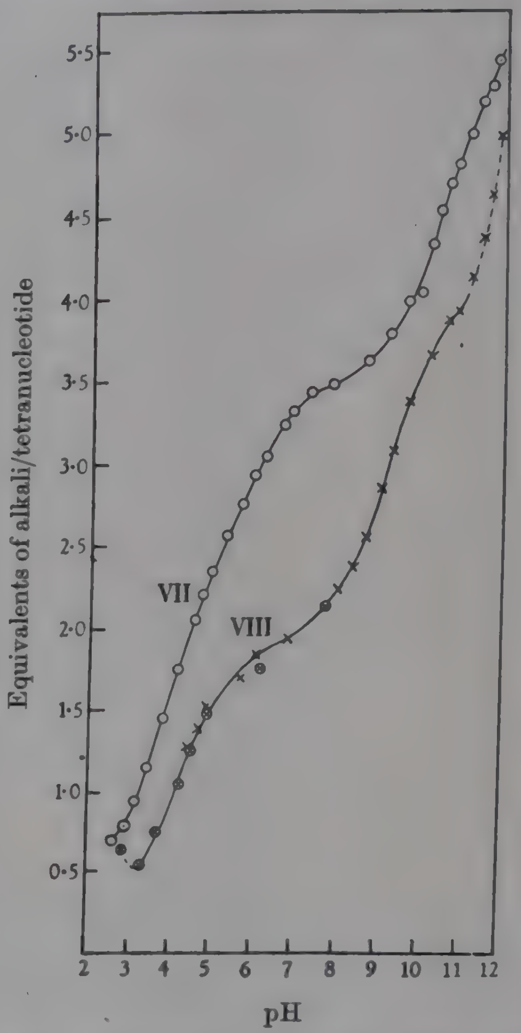

Fig. 3. The electrometric titration of yeast ribonucleic acid (Y 5/42), curve VII, ⊙, and its *H* product (HY 7/47 B), curve VIII, ×⊗.

Table 3. *Numbers of titratable groups/tetranucleotide present in nucleic acids and numbers of groups titratable or blocked in their H compounds*

	Primary phosphoryl	Secondary phosphoryl	Amino	Purine-pyrimidine hydroxyl	Total groups blocked	$(-CH_2-CH_2)_2S$ residues*	$HOCH_2.CH_2-S-CH_2-CH_2-$ residues*
YNA, Y 5/42	2·8	0·8	2·7–3·0	2·0	—	—	—
H-YNA, HY 7/47 B	1·6	0·4	$\not<$ 1·2	1·8	—	—	—
Groups blocked	1·2	0·4	0–1·8	0·2	1·8–3·6	2·4	2·5
DYNA, DY 8	2·5	1·2†	0	3·0	—	—	—
H-DYNA, HDY 2/34 B	1·2	0·6†	0	3·0	—	—	—
Groups blocked	1·3	0·6†	0	0	1·9	2·0	2·1
TNA, T 5	4·0	0	3·0	2·0	—	—	—
H-TNA, HT 3/53 B	1·4	0·7‡	$\not<$ 1·4	1·0	—	—	—
Groups blocked	2·6	(–0·7)	0–1·6	1·0	3·6–5·2	2·2	2·2
Guanylic acid	1·0	1·0	1·0	1·0	—	—	—
H-guanylic acid HG 4/71	0·8	0·55	0·1	0·5	—	—	—
Groups blocked	0·2	0·45	0·9	0·5	2·05	1·85	2·03

YNA = yeast nucleic acid; DYNA = deaminated yeast nucleic acid; TNA = thymonucleic acid.
* Calculated from sulphur analyses; see text. † Includes hydroxyl of xanthine. ‡ See text.

The titration curve of the B product from deoxypentose nucleic acid (T5) of high molecular weight is compared in Fig. 2 with that of a sample of the parent acid which has been treated with mineral acid; this procedure was adopted because the curve for the acid-treated substance differs slightly from that of the original (Gulland, Jordan & Taylor, 1947), and because the B compound was obtained by precipitation with mineral acid. The identity of the 0·7 equivalents of a group titrating between pH 6·0 and 8·4 is obscure; the only likely group titrating in this range is a secondary phosphoryl group, but the dissociation constant of the liberated group ($pK'_a = 7{\cdot}2$) is different from that of the secondary phosphoryl group in thymic acid ($pK'_a = 6{\cdot}4$) (Gulland, Jordan & Taylor, 1947) with which it may suitably be compared.

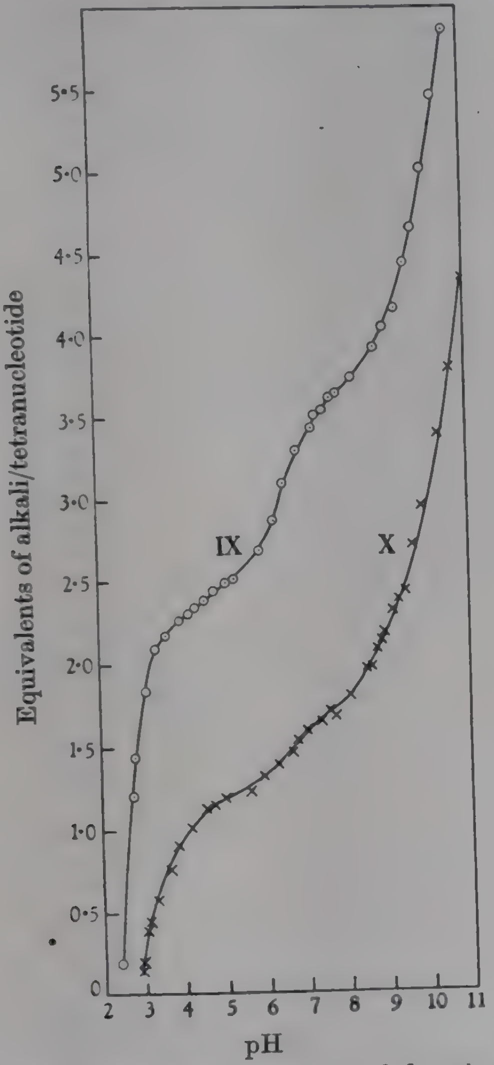

Fig. 4. The electrometric titration of deaminated yeast ribonucleic (DY8), curve IX, ⊙, and its *H* product (HDY2/34B), curve X, ×.

The dissociation constant of the purine-pyrimidine hydroxyl groups of yeast ribonucleic acid is changed from $pK'_a = 10{\cdot}5$ to $9{\cdot}4$ by interaction of the acid with *H*. Such changes are not yet fully understood and have been discussed elsewhere (Fletcher, Gulland & Jordan, 1944; Gulland, Jordan & Taylor, 1947).

For the purpose of calculating the number of purine hydroxyl groups in *H*-guanylic acid, it is assumed that the dissociation constant of these is the same ($pK'_a = 9{\cdot}5$) as that of guanylic acid; owing to the extensive removal of *H* residues by hydrolysis above pH 10, it was not possible to determine the complete titration curve.

The carbohydrate hydroxyl groups do not titrate below pH 12, and hence it was not possible to determine the extent, if any, of their reaction with *H* by electrometric titration.

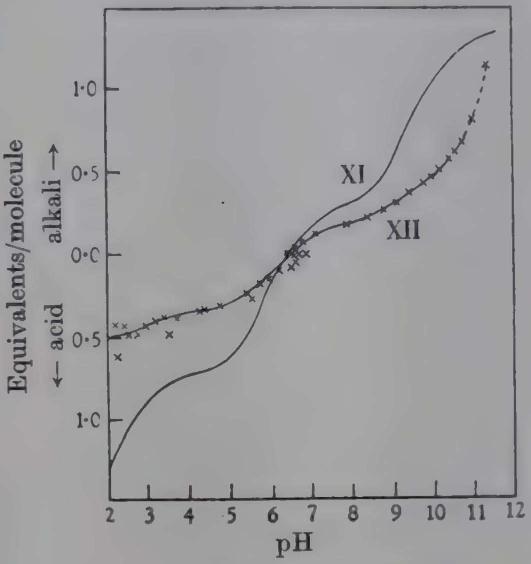

Fig. 5. The electrometric titration of guanylic acid, curve XI (theoretical curve), and its *H* product (HG4/71), curve XII, ×.

It is not possible to determine by titration alone the extent of the reaction of *H* with the primary phosphoryl group of guanylic acid. This can be calculated, however, on the assumption that no phosphorus is lost during the reaction. Analysis of the *H*-guanylic acid shows that the atom ratio P : Na is 1 : 1·2; thus there are in all 1·2 primary and secondary phosphoryl groups neutralized at pH 6·4 which is the pH of the *H*-guanylic acid solution in water. On titration with alkali (curve XII, Fig. 5) a further 0·15 equivalent of a secondary phosphoryl group is titrated. The total number of secondary and primary phosphoryl groups which have not reacted with *H* is thus 1·35. Since the titration shows that of these, 0·55 equivalent is due to secondary phosphoryl dissociation, it follows that 0·8 equivalent of a primary phosphoryl group has not reacted with *H* and hence 0·2 group has reacted. The total number of titratable groups blocked is 2·05.

DISCUSSION

Nucleic acids contain various groups which might be expected under suitable conditions to react with *H*. Assuming the generally accepted polynucleotide structure of the acids, the number and nature of these groups are summarized in Table 3, which is based on the polymerization of a statistical tetranucleotide.

Of the various types of reaction into which *H* can enter, that in which the chlorine atoms are replaced by other substituents seems the most probable in the present circumstances, i.e. of the Hughes-Ingold S_N1 type (Watson, 1938; Hughes, 1941). *H* enters into such reactions with amino groups (Clarke, 1912; Helfrich & Reid, 1920; Cashmore & McCombie, 1923; Lawson & Reid, 1925; Ford-Moore, 1940; Boursnell, Francis & Wormall, 1946; Moore, Stein & Fruton, 1946), phenolic anions (Helfrich & Reid, 1920; Ford-Moore, 1940) and carboxylic anions (Doering, Linstead & Ball, 1942; Moore *et al.* 1946), but not with the undissociated carboxyl group (Cohen & Harris, 1942). Inspection of the titration curves of nucleic acids and nucleotides shows that in solution at pH 7 the phosphoryl groups are in the ionic form and the amino groups undissociated, and are thus likely to react with *H*. The purine-pyrimidine hydroxyls ($\text{—N}=\overset{|}{\text{C}}\text{—OH}$) titrate over the range pH 8·0–11·5, being thus similar to phenols in character, and would not be expected to react at pH 7. In DYNA, one hydroxyl group of xanthine is partly dissociated at pH 7 and should therefore react with *H*, whereas the other hydroxyl does not titrate at reactions more acid than pH 12. The sugar hydroxyls have $pK'_a = 13$ and would therefore not be expected to react at pH 7.

The number of titratable groups blocked by *H* is compared with the number of *H* residues calculated from the sulphur analyses (Table 3). It will be seen that in general the results agree with those predicted on theoretical grounds, although the numbers of groups reacting could not always be precisely determined. The only marked divergence is the reaction of the purine-pyrimidine hydroxyl in TNA and guanylic acid; this was unexpected but no satisfactory explanation can be offered.

In the B product from DYNA (HDY 2/34 B) and in the *H* compound from guanylic acid (HG 4/71), it is evident that within experimental error the number of *H* residues is equal to the total number of titratable groups blocked. It is therefore concluded that each titratable group blocked is substituted by one *H* residue. It seems probable, although not certain, that this residue is in the form

$$\text{—CH}_2.\text{CH}_2.\text{S}.\text{CH}_2.\text{CH}_2.\text{OH},$$

since none of the products contained chlorine. It is considered unlikely that *H* has attacked simultaneously a titratable group and a non-titratable hydroxyl, because these latter would not be expected to undergo alkylation at pH 7. Two criticisms of this conclusion should be mentioned, although neither is regarded as likely to be valid. First, it is possible that half of the reacting *H* molecules have cross-linked non-titratable groups and the other half entered into cross linkages with titratable groups; such circumstances, although in agreement with the analytical results, are considered not only to be statistically improbable but also unlikely to occur at pH 7. Secondly, there is the possibility that the reactions with *H* are more complex in character and resemble the formation and decomposition of sulphonium salts known to occur between *H* and its substituted derivatives (Bell, Bennett & Hock, 1927). This reaction mechanism is considered to be most improbable since the formation of sulphonium salts is exceedingly slow at room temperature, and also since no 'sulphonium' dissociation was detected by electrometric titration. It is also possible that *H* has reacted with the amino groups to form 1:4-thiazan derivatives, a type of reaction observed under more vigorous conditions by Clarke (1912), Helfrich & Reid (1920), Lawson & Reid (1925) and Boursnell *et al.* (1946). Our data do not permit a decision as to the nature of the reaction undergone by the amino groups, and it is noteworthy that Moore *et al.* (1946) were unable to establish whether the amino groups of amino-acids and peptides formed secondary amines or thiazans in their reaction with *H*.

The precise figure for the total number of groups blocked in *H*-YNA (HY 7/47 B) could not be ascertained by electrometric titration, but since the number of the primary and secondary phosphoryl and purine-pyrimidine hydroxyl groups blocked is similar to that in *H*-DYNA (HDY 2/34 B), it appears unlikely that any extensive cross linkage has occurred.

In the B product from TNA, on the other hand, it is clear that the number of blocked titratable groups is greater than the number of *H* residues in the molecule, indicating that *H* molecules have reacted with two titratable groups, either in the same molecule or by a process of cross linking between polynucleotide chains. The comparison of viscosities of solutions of TNA before and after treatment with *H* provides additional evidence in favour of some measure of intermolecular cross linkage in view of the increased viscosity of the treated sample.

The calculated analytical and titration data for complete and no cross linking by *H* are compared with the observed data for *H*-guanylic acid in Table 4. The evidence confirms the theory that each titratable group is attached to one *H* residue and that there is little or no cross linking. Such a comparison is not satisfactory in the case of the nucleic acids as the calculated values for complete cross linking and no cross linking are too close together to permit distinction by analysis and titration.

The formation of two series of products, not one, by the interaction of nucleic acids with *H* at pH 7 must now be considered. The salient facts are as follows: (i) both A and B products were free from chlorine and contained unsubstituted phosphoryl groups, the members of the A series being sparingly soluble sodium salts (Table 2), dissolving only at reactions more alkaline than pH 10, whereas the B products were precipitated by mineral acid from solutions of their sodium salts; (ii) the ratio 'yield of A/yield of B' in any given instance increased with longer duration of reaction and higher molecular ratios of *H*/tetranucleotide, but was practically independent of the volume of the reaction mixture (Table 1); (iii) there was no marked or constant difference in the sulphur contents of the A and B products (Table 2); (iv) further treatment of a B compound gave rise to a new pair of A and B products, both containing considerably more sulphur (Table 2, HHDY 3/43 A/B). It is therefore clear that related members of the A and B series were not identical, and that the B products were not direct precursors of the A products formed simultaneously and having approximately the same sulphur content.

Table 4. *Comparison of observed and calculated data for H-guanylic acid* (*HG*4/71)

Analysis	Calculated for no cross linkage	Calculated for complete cross linkage	Observed
No. of groups blocked/mol.	2·03	1·85	2·05
(%)			
C	36·2	38·1	37·0
H	4·9	4·9	4·7
N	11·7	12·8	11·8
P	5·2	5·7	5·3
S	10·8	10·8	10·8
Na	4·6	5·0	4·7

Two explanations of these differences may be mentioned, and these could operate singly or simultaneously. It is possible that the A products have been produced by the cross linking by *H* of lyophilic groups on the same polynucleotide chain; thus two solubility-conferring groups would be rendered inactive for each atom of sulphur introduced. Alternatively, the A products may have a much higher molecular weight than the B products as a result of the cross linking occurring between different polynucleotide chains. The B products are considered to be mainly the monosubstituted derivatives of thiodiglycol (see above). These hypotheses are in conformity with the possibility that B compounds on further treatment with *H* can produce either B compounds of higher sulphur content or by cross linking, A compounds of higher sulphur content (see (ii) and (iv) above).

There is thus ample evidence that at the pH values pertaining in tissues both deoxypentose nucleic acid of calf thymus and yeast ribonucleic acid will react with *H*. It is to be emphasized that it is not known whether the structure of pentose and deoxypentose nucleic acids of all tissues are the same as or different from those of YNA and TNA (Gulland, 1944, 1947; Gulland *et al.* 1945; Davidson & Waymouth, 1944–5), but there is no evidence to suggest that the reactive groups present in nucleic acids from other sources are not similar to or identical with those of YNA and TNA. The biological implications of the results are clear. It is generally agreed that deoxypentose and pentose nucleic acids are constituents of all nuclei, and are involved in the mechanism of mitosis. The attack of cell nucleic acids by *H* would interfere with this mechanism and hence with the proliferation of tissues injured by *H*. The present investigation thus provides a chemical parallel to and possibly an explanation of the cytological observations of Koller (1942), Robson (1943) and Fell & Allsopp (1943).

SUMMARY

1. It has been found possible to induce reaction between nucleic acids, guanylic acid and mustard gas (*H*) under mild conditions of pH and temperature.

2. The products of the reaction between nucleic acids and mustard gas have been isolated and analyzed. They are found to contain sulphur but not chlorine.

3. Electrometric titration of the *H* compounds shows that the primary and secondary phosphoryl groups, and the amino groups have in every case, when present, reacted with *H*. The purine-pyrimidine hydroxyl groups have been found to react only in certain cases.

4. The viscosity of solutions of the *H* compound of a high molecular weight sample of deoxypentose nucleic acid was found to be much greater than that of the original acid.

5. Possible structures of the *H* compounds are discussed, and the significance of the results considered in relation to the delayed healing of mustard gas lesions.

It is a pleasure to record our thanks to the Chief Scientist, Ministry of Supply, for permission to publish these results; to Mr J. E. Still for performing a number of the microanalyses; to Mr J. M. Creeth for the determinations of viscosities; to Mr W. E. Fletcher for the titration curve of DY 8; to Imperial Chemical Industries Ltd. for the loan of apparatus; and to the British Empire Cancer Campaign for a maintenance grant to one of us (D. T. E.) during a short period at the end of the work here recorded.

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The Component Acids of Herring Visceral Fat

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The component acids of North Sea herring flesh fats laid down at different seasons, including those of intensive feeding when much fat is deposited, and those of relative inanition when the body fat content falls to a low level, have been discussed by Lovern (1938). Similar data for the flesh fat of Icelandic herring have been given by Bjarnason & Meara (1944). The component acids of fats from other parts of the herring have not been studied in detail hitherto. Some time ago Mr J. R. Bruce, of the Marine Biological Station, Port Erin, Isle of Man, sent to us a quantity of oil extracted from the viscera of herrings taken locally, and we have now had an opportunity to examine the nature of the component acids of the visceral fat, which displays interesting differences from the characteristic composition of herring flesh fats.

METHODS

The visceral oil studied was from herrings caught off the south-western coast of the Isle of Man during the first week of August, 1942. The herrings had been gutted by hand in a local factory prior to conversion into kippers. The offal would have contained intestines, stomach, liver, mesentery, etc., and also the milt or roe, together with a very small number of heads and of broken fish (these forming, however, only a minute percentage of the whole). It was rendered by boiling with water in the Port Erin laboratory. The separated oil was filtered and then sent to us. It was stored in a full bottle in a refrigerator for a long period before we had an opportunity to examine it, but had remained fresh and free from oxidation. The oil was pale yellow in colour, had saponification equivalent 296·2 and iodine value 146·8, and contained 5·7% of free fatty acid (as oleic) and 0·75% of unsaponifiable matter.

Its component acids were determined by fractional distillation in a vacuum of their methyl esters through an electrically-heated packed column. Before conversion to esters the mixed acids from 205 g. of the oil were first resolved into three groups by crystallization from acetone and from ether at low temperature. It has been shown recently (Hilditch & Pathak, 1947) that low temperature crystallization from solvents affords much more rapid and efficient preliminary separation of the mixed acids of marine animal oils than the procedures hitherto employed, in which use was made successively of the different solubility of their lithium salts in acetone and of their lead salts in ethanol (Hilditch & Maddison, 1942; Bjarnason & Meara, 1944).

In the present instance the herring visceral oil mixed fatty acids (190 g.), after removal of the bulk of the unsaponifiable matter present, were first crystallized from acetone (1900 ml.) at −60° for 5 hr., when 66·9 g. (iodine value 277·0) remained in solution; recrystallization of the deposited acids under the same conditions left a further 17·7 g. (iodine value 236·1) in solution. These two soluble portions were united as fraction *C* (84·6 g., iodine value 268·4). The portion (105 g.) which had remained insoluble in acetone at −60° was then crystallized from ether (1050 ml.) at −30° for 5 hr. when 53·8 g. (*B*, iodine value 89·8) remained in solution and 51·2 g. (*A*, iodine value 23·9) deposited. Nearly all the polyethenoid constituents of the acids were thus obtained in the most soluble portion *C*, whilst the low iodine value of the least soluble portion *A* indicated that only monoethenoid acids were present therein:

Fraction		(g.)	(%)	Iodine value
A	Insoluble in ether at −30°	51·2	27·0	23·9
B	Soluble in ether at −30°	53·8	28·4	89·8
C	Soluble in acetone at −60°	84·6	44·6	268·4

Each group of acids was separately converted into methyl esters, which were fractionally distilled as described above, the most unsaturated group *C* being dealt with first, fractionation and analysis of the ester fractions being carried out as rapidly as possible to minimize chances of autoxidation and polymerization. From the iodine values and equivalents of the ester fractions the composition of the latter was calculated by methods which have been described elsewhere (Hilditch, 1947). The mean unsaturation, expressed by the fractional number of hydrogen atoms short of saturation, e.g. −2·0 (monoethenoid), was determined as usual by interpolation or extrapolation from the respective ester fractions in each of the groups *B* and *C*, from which the mean equivalent of each of the homologous ester groups (C_{16}, C_{18}, C_{20} and C_{22}) followed (the values employed are listed in Table 2).

RESULTS

The ester-fractionation data obtained for each of the three groups of herring visceral oil fatty acids are summarized in Table 1.

Table 1. *Fractionation of methyl esters of herring visceral oil acids A, B and C*

Fraction	(g.)	Saponification equivalent	Iodine value
	Methyl esters of acids *A*		
A 1	2·50	246·0	0·8
A 2	2·64	250·8	0·9
A 3	3·68	263·6	0·9
A 4	3·89	268·0	1·0
A 5	4·72	268·3	1·2
A 6	5·41	268·5	1·3
A 7	4·44	268·9	1·5
A 8	4·23	270·5	3·0
A 9	4·30	292·0	29·1
A 10	3·74	301·6	58·3
A 11	4·45	302·1	71·3
A 12	4·95	310·9*	68·7
	48·95		
	Methyl esters of acids *B*		
B 1	3·34	247·8	5·4
B 2	3·59	252·8	31·4
B 3	4·56	281·3	73·1
B 4	4·65	292·3	87·9
B 5	4·72	293·3	88·9
B 6	5·10	297·7	93·8
B 7	4·83	299·1	102·3
B 8	4·94	301·3	107·0
B 9	5·13	302·1	113·7
B 10	4·36	303·5	118·2
B 11	2·25	305·9	115·4
B 12	3·28	320·9*	112·2
	50·75		
	Methyl esters of acids *C*		
C 1	3·40	250·5	85·9
C 2	3·94	266·4	123·8
C 3	3·97	271·4	128·8
C 4	3·82	277·8	134·4
C 5	4·04	290·1	156·7
C 6	5·44	301·8	158·2
C 7	5·38	307·5	212·2
C 8	6·24	313·5	318·4
C 9	5·20	319·4	346·4
C 10	4·86	327·3	362·9
C 11	4·03	332·4	377·4
C 12	5·31	335·9	371·8
C 13	4·47	351·4*	308·7
	60·10		

* Equivalents of esters (freed from unsaponifiable matter): *A* 12, 307·4; *B* 12, 314·8; *C* 13, 340·8.

Table 2. *Equivalents, iodine values and mean unsaturation employed in calculating the composition of unsaturated methyl-ester fractions from groups B and C*

Methyl esters of acids	*B* Saponification equivalent	*B* Iodine value	*B* Mean unsaturation	*C* Saponification equivalent	*C* Iodine value	*C* Mean unsaturation
C_{14}	240·0	105·8	−2·0	240·0	105·8	− 2·0
C_{16}	268·0	94·8	−2·0	267·4	123·5	− 2·6
C_{18}	295·9	90·1	−2·1	294·3	159·7	− 3·7
C_{20}	321·3	185·8	−4·7	317·6	335·0	− 8·4
C_{22}	—	—	—	343·5	388·3	−10·5

The percentage composition (by weight) of each group of acids *A*, *B*, *C* was then calculated (Table 3) and therefrom that of the total fatty acids of the herring intestinal oil can be obtained (Table 4).

Table 3. *Composition (% w/w) of the acids in groups A, B and C*

Acid	*A* (%)	*B* (%)	*C* (%)
Myristic	11·2	7·6	1·4
Palmitic	53·1	4·6	—
Stearic	9·2	1·2	—
Arachidic	1·2	—	—
Unsaturated C_{14}	Trace	1·4	2·3
Unsaturated C_{16}	1·2	6·5	18·6
Unsaturated C_{18}	17·5	62·3	20·8
Unsaturated C_{20}	6·5	16·3	35·9
Unsaturated C_{22}	—	—	20·8
Unsaponifiable	0·1	0·1	0·2

Table 4. *Component acids of herring visceral oil*

Acid	*A* (27·0%)	*B* (28·4%)	*C* (44·6%)	Total	Fatty acids (%, w/w)	Fatty acids (%, by mol.)
Myristic	3·0	2·2	0·6	5·8	5·8	7·1
Palmitic	14·4	1·3	—	15·7	15·7	17·0
Stearic	2·5	0·3	—	2·8	2·8	2·7
Arachidic	0·3	—	—	0·3	0·3	0·3
Unsaturated C_{14}	—	0·4	1·0	1·4 (− 2·0)	1·4	1·7
Unsaturated C_{16}	0·3	1·9	8·3	10·5 (− 2·5)	10·5	11·5
Unsaturated C_{18}	4·7	17·7	9·3	31·7 (− 2·6)	31·8	31·4
Unsaturated C_{20}	1·8	4·6	16·0	22·4 (− 7·1)	22·4	20·4
Unsaturated C_{22}	—	—	9·3	9·3 (−10·5)	9·3	7·9
Unsaponifiable	Trace	Trace	0·1	0·1	—	—

DISCUSSION

The composition of the herring visceral oil shows marked differences both from that of body (flesh) fats of North Sea herrings (Lovern, 1938) and of Iceland herrings (Bjarnason & Meara, 1944), and from that of the fat in one of the main foods of the herring, the copepod *Calanus finmarchicus* (Lovern, 1935). These differences are illustrated in Table 5.

Mr W. C. Smith, of Port Erin Marine Biological Station, has informed us that *Calanus* is an important item in the diet of herring in Manx waters, and also reminded us that of course the fish do not feed actively at or near the spawning season (early autumn).

The circumstance that the specimen of visceral fat studied probably contained an uncertain amount of herring milt and roe introduces a possibly complicating factor, since Lovern (private communication to the authors) has observed that herring roe glycerides have iodine values of 170 and somewhat higher, and thus may contain large proportions of C_{20} and C_{22} acids of high unsaturation (Lovern, unfortunately, was unable to undertake full analyses of the roe component acids). Since, however, Manx herring at the time of year concerned often include a fair proportion of immature fish, it is on the whole likely that the proportion of milt and roe fat in the total visceral fat which we have examined may not have been very substantial, and that the component acid data in Table 5 represent for the most part those for the fats of the herring viscera.

The chief differences between these visceral fatty acids and those of the herring body fats are in the much lower content of unsaturated C_{22} acids in the former, coupled with much higher mean unsaturation of the acids of both the C_{22} and C_{20} series as compared with those of the body fats; further, the proportion of unsaturated C_{18} acids is much larger in the visceral than in the body fats, although their mean unsaturation is considerably lower. Subordinate differences may include a somewhat lower content of unsaturated C_{20} acids, and somewhat higher contents of total saturated acids and, perhaps, of unsaturated C_{16} acids as well as of palmitic acid.

Table 5. *Component acids (%, w/w) of herring body and visceral fats and of copepod fat*

	Body (flesh) fats				
	North Sea			Visceral fat Isle of Man (August)	Copepod fat (*Calanus finmarchicus*)
	July	October	Iceland		
Iodine value	152·5	138·6	140·0	146·8	177·6
Component acids:	(%)	(%)	(%)	(%)	(%)
Myristic	8·3	7·3	7·1	5·8	8·3
Palmitic	12·1	13·0	11·7	15·7	10·6
Stearic	0·3	Trace	0·8	2·8	1·3
Arachidic	—	—	0·1	0·3	—
Unsaturated C_{14}	0·5	0·8	1·2	1·4	1·6
Unsaturated C_{16}	6·4	4·9	11·8	10·5	11·8
Unsaturated C_{18}	21·0	20·7	19·6	31·8	16·8
Unsaturated C_{20}	28·3	30·1	25·9	22·4	24·5
Unsaturated C_{22}	23·1	23·2	21·7	9·3	25·1
Mean unsaturation of:					
Unsaturated C_{14}	−2·0	−2·0	−2·0	−2·0	−2·0
Unsaturated C_{16}	−3·4	−2·7	−2·4	−2·5	−2·4
Unsaturated C_{18}	−4·5	−4·2	−3·5	−2·6	−5·1
Unsaturated C_{20}	−5·5	−4·6	−5·2	−7·1	−7·8
Unsaturated C_{22}	−4·6	−4·3	−4·3	−10·5	−8·1

Lovern (1938) has suggested that characteristics of herring body fats include an unusually high content of unsaturated C_{22} acids, unusually low unsaturation in the C_{20} and C_{22} acids, and unusually high unsaturation in the C_{16} and C_{18} acids, with lower contents than usual of unsaturated C_{16} acids (although the amounts of total C_{16} acids fall within the usual range). These conclusions were based on his previous statistical survey of the average composition of marine fish fats (Lovern, 1937), in which, however, the data referred to many more liver fats than body fats from marine fish. The statistical data indicated the following approximate amounts and unsaturation of unsaturated acids as being characteristic for marine fish in general:

	(%)	Mean unsaturation
Unsaturated C_{16}	10	−2·0 to −2·5
Unsaturated C_{18}	25	−2·6 to −3·3
Unsaturated C_{20}	25	−5·5 to −6·5
Unsaturated C_{22}	15	−7·0 to −9·5

Turning to the herring visceral fat now under discussion, it will be seen that its component acids align themselves much more closely, both in their proportions and in their mean unsaturation, with Lovern's 'average values' for marine fish fats than with the observed values for herring flesh (depot) fats. The mean unsaturation for the various groups falls within or approximates to Lovern's average limits in each case, whilst the only significant differences in proportions of acids present are a larger amount of unsaturated C_{18} and a smaller amount of unsaturated C_{22} acids.

On the other hand, although the mean unsaturation of the various groups (except in the C_{18} acids) is much the same in herring visceral fat and in the dietary *Calanus* fat, the respective differences in content of unsaturated C_{18} and unsaturated C_{22} acids are as well marked, or even more so, than those between the same groups of acids in the herring visceral and body fats. Lovern (1938) has put forward the view that the herring body fat, with component acids in much the same proportions as in *Calanus* fat, has been stored as a result of ingestion in quantity in the herring diet of the latter fat, but that prior to laying down in the herring fat depots the *Calanus* fat has been modified by considerable hydrogenation of its unsaturated C_{20} and C_{22} components and some dehydrogenation of its unsaturated C_{16} components.

At first sight the observed composition of the herring visceral fat would seem to be at variance with Lovern's explanation, but in our opinion this does not necessarily follow. The visceral fat may be considered, in the herring, to be more of the nature of an organ fat than a depot fat; indeed, its similarity in composition to the 'average marine fish fats' (which as already pointed out were mainly liver fats) may be due to this circumstance. (Fish livers, of course, often function simultaneously as depots, but very broadly speaking the main characteristics of the component acids of fish liver fats are similar, whether the liver is rich or poor in fat.) If the visceral fat of the herring is considered to be an organ fat rather than a depot fat, its dissimilarity to the specific depot fat laid down by the herring is not necessarily surprising. Thus the liver lipids of land animals (pig, sheep, ox) which have been examined in detail are found, either in their glycerides or their phosphatides, to be respectively broadly similar in composition (Hilditch & Shorland, 1937), and to have some features (e.g. presence of unsaturated C_{16}, C_{20} and C_{22} acids) which are not only common to all these animal liver lipids but are also reminiscent of the glyceride fats of aquatic organisms: but neither the glyceride nor the phosphatide fractions of these liver lipids have any resemblance to the depot fats laid down by the different animals, the depot fats of which moreover differ considerably in composition from one species to another.

We therefore confine ourselves to the conclusion that our present results show that herring visceral fat differs materially in composition from the typical fat laid down in the herring fat depots, and that its component acids are very similar in content and in their degree of unsaturation to those of what appear to be typical marine fish liver fats. It is also evident that much more data could still profitably be sought as to the detailed composition of the fatty acids in different organs and depots from a wide range of species of fish.

SUMMARY

1. The component acids of herring visceral fat have been determined by the ester-fractionation procedure.

2. Preliminary resolution of marine animal fatty acids into groups differing in unsaturation, by means of crystallization from suitable solvents at low temperatures, is shown to be preferable to their separation in the form of lithium and lead salts.

3. The herring visceral oil fatty acids differ from those of herring body fats (and of *Calanus* fat, a fat largely ingested by the herring) in their much lower content of unsaturated C_{22} acids and much higher content of unsaturated C_{18} acids, together with much higher degree of average unsaturation of the C_{20} and C_{22} acids and lower average unsaturation of the C_{18} acids.

We wish to express our thanks to Mr J. R. Bruce for affording us the opportunity to study the specimen of herring visceral fat.

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Obituary Notice

WALTER RAMSDEN, 1868–1947

Walter Ramsden, whose death was announced on 27 March 1947, was an original member of the Biochemical Society. His loss will be felt keenly by his many friends, both on account of his amiable personality and of his scientific interests.

The fourth son of W. H. F. Ramsden, a physician of Saddleworth, Yorkshire, and of Catherine Kay, he was born on 4 October, 1868. At the age of 14, owing to trouble with his eyes, he left Manchester Grammar School, which he had entered when twelve years old, to study privately for four years at home. He came to Oxford with an Exhibition at Keble College, and obtained a 1st Class in the Honour School of Physiology in 1892. He was Radcliffe Travelling Fellow from 1893 to 1896, and during this time he studied at Zürich and Vienna and finally at Guy's Hospital, taking his Final M.B., B.Ch. in 1897 and his M.D. in 1902. Never married and never in medical practice, he devoted himself to teaching and research.

In 1899 he was elected at Pembroke College to the Sheppard Medical Fellowship which he held to the end of his life. It is interesting to note that under the old statutes this Fellowship is tenable for life unless forfeited by marriage. His talents were soon utilized in the Department of Physiology where he became Lecturer in Physiological Chemistry under Prof. Gotch. He held the post until 1914, and during his last year, he worked under Prof. Sherrington who had succeeded Gotch. He took part in other University activities and exercised a stimulating influence on his pupils; Edward Whitley, for instance, later became with Benjamin Moore the joint founder of the *Biochemical Journal*, and subsequently started Biochemistry in Oxford with the endowment of the Whitley Chair, Moore being the first holder.

From Oxford Ramsden went in 1914 to Liverpool to become Johnston Professor of Biochemistry in succession to Benjamin Moore. The effect made in Liverpool by his personal charm is well described in a recent obituary notice (*Nature, Lond.*, 1947, **159**, 801).

In 1931 he retired to devote his remaining years to research in Oxford. For the first few years he worked in the Department of Pathology and from 1937 in the Department of Biochemistry. He pursued his researches on proteins from the silkworm until the end, so far as failing health and a troublesome gastric condition would permit. For many years he had not enjoyed good health, but nothing interfered with the charm of his old world courtesy. His ready reception of new ideas and willingness to discuss them remained as characteristic as his fund of anecdotes. Many will retain the vivid impression of his appearance in the appropriate season with a tin containing a kindly gift of mulberries.

Those who knew him early in his career, and appreciated his brilliant skill as an experimenter in the field of surface chemistry, were disappointed that his early promise did not materialize with sufficient solidity in print. Though he was ever willing to discuss his work and ideas with friends, a certain fastidiousness and a wish to obtain more thorough completion, inhibited the published word. Nevertheless the work which appeared was important. He was among the earlier biochemists to appreciate the importance of physico-chemical surface studies in biology. His first researches set the stage for his life's devotion to the phenomena of surfaces in their especial relation to protein chemistry; in 'Die Coagulierung von Eiweisskörpern auf mech. Weg' (*Arch. Anat. Physiol., Lpz.*, 1894, pp. 517–34), he showed how denaturation of proteins occurred at the interfaces formed by mechanical shaking; this was to be followed later by work on the 'separation of solids in surface layers of solutions' (*Proc. Roy. Soc.* B, 1903, **72**, 156; also *Z. phys. Chem.* 1904, **47**, 336). The latter work is not as well known as it should be and has a pioneer relationship to some modern protein film work. In 1902 (*Proc. Physiol. Soc.* 5 July) he described some new properties of urea; proteins were dissolved by urea with denaturation; coagulated proteins went into solution, and even a 'dead frog placed in saturated urea solution becomes translucent and falls to pieces in a few hours'; 'thiourea appears to have precisely the same effects as urea'.

This pioneer work paved the way for more recent investigations with urea and guanidine acting on proteins. With N. G. Chavasse, in the effect on viscosities of denatured egg albumin solutions, it was followed in detail and formed a contribution to a Faraday Society Discussion in March 1913 (also *Z. Chem. Industr. Kolloide*, 1913, **12**, 250), and further details were published with J. Hatton (*Chem. and Ind.* 1930, **8**, 851); Ramsden showed, for instance, that urea unmasked the thiol and disulphide groups in egg albumin and added details upon the rate of denaturation. During World War I, he worked with colleagues upon the estimation of quinine (see Ramsden, *Brit. med. J.*

1920, **2**, 117; papers in *Ann. trop. Med. Parasit.* **11**, **12** and **13**). This investigation occupied much time and the details proved troublesome. After this interlude, he soon returned to surfaces, and a more fully developed account of his theories was given in 1923 (Presidential address on Surfaces and Films to the Liverpool Biological Society; 'Demonstrations in Interfacial Phenomena at British Association Meeting', *Nature, Lond.*, 1923, **112**, 671).

There followed in 1926 (*Trans. Faraday Soc.* **22**, 484) a fuller account of part of his theories in a discussion ('Some Physical Properties of Composite Surfaces'), embodying work carried out with R. H. Hurst (see also *J. Soc. Chem. Ind., Lond.*, 1929, **15**, 1113). In particular, it was noticed that some insoluble liquids or water-soluble colloids (saponin), would spread to enormous areas upon pure water surfaces. This led to his conception of 'disturbance haloes', and to his insistence upon the reality of surface tensions. He always argued that it was erroneous to think Langmuir's apparatus measured a 'surface pressure' of the film particles upon the movable barrier, and preferred to consider them as produced by decrements in the respective tensions. This he held very strongly and hated the thought that there should be no reality in surface tension. In 1927, Ramsden described in Clayton's 'Theory of Emulsions and their technical treatment' his theory of emulsions stabilized by solid particles, in which characteristically he stated that with J. Brooks, he had devoted two years from 1922 to 1924 to an experimental test by numerous observations of the contact angle made against a variety of solids at water-benzene or paraffin interfaces. To the writer's knowledge, these data have never been published, though some of the complicated solubility relations holding with cholesterol for mixtures of kephalin and lecithin in systems containing water and a fat solvent were briefly described (Brooks, J., Ramsden, W. & Earlam, L. (1929), *J. Soc. Chem. Ind., Lond.*, **15**, 1113).

About 1930 there followed an introduction to work which occupied him in his retirement. A deeper investigation of the reactions given by sheep's wool, treated with aqueous urea solutions, led to the finding of a pseudo thiol reaction given with an alkaline urea solution and an 'aged' nitroprusside solution, and to some qualification of a previous statement. It is possible that this work upon wool led to the prolonged researches upon fibroinogen; how the stiff water-soluble fibroinogen in the silk depots (larva of *Bombyx mori*) is transformed into the insoluble fibroin strands of silk (*Nature, Lond.*, 1938, **142**, 1120).

Briefly the interesting new fact emerged that the protein fibroinogen, which is thermostable, is coagulated by shearing between two flat plates of glass, and by freezing. The presence of certain salts prevents the latter change. Interestingly enough, this coagulation by shearing was not a case of surface coagulation similar to that described by himself over 40 years earlier. Other details about this phenomenon were added later, at a Biochemical Society Meeting in Oxford (26 October) during World War II (*J. Soc. Chem. Ind., Lond.*, 1940, **59**, 730). At the time of his death, he was trying to confirm an important suggestion that a catalytic factor might be concerned.

This brief account serves to illustrate a life-long interest, but cannot describe at all adequately the influence of his earnest spirit of inquiry, and his refusal to let ill-health divert him from his interests. He had many hobbies; he was a strong advocate of Esperanto and was active in spreading that gospel. In spite of his poor health, up to within four months of his decease, he would cheerfully ride 20 to 30 miles in hilly country on his bicycle, and it was remarkable how easily he negotiated the now heavily congested roads between the Parks and Pembroke College.

(The writer gratefully acknowledges help from Sir Charles Sherrington and Mr J. R. P. O'Brien in the preparation of this account.)

R. A. PETERS

Studies in the Biochemistry of Micro-organisms

76. MYCELIANAMIDE, $C_{22}H_{28}O_5N_2$, A METABOLIC PRODUCT OF *PENICILLIUM GRISEO-FULVUM* DIERCKX. PART 1. PREPARATION, PROPERTIES, AND BREAKDOWN PRODUCTS

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(*Received 5 August* 1947)

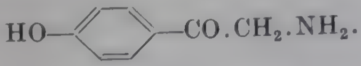
The occurrence of a crystalline, ether-soluble, nitrogenous compound in the mycelium of *Penicillium griseo-fulvum* Dierckx, grown on synthetic media, has previously been mentioned in several studies from this laboratory on the metabolic products of this mould. The compound was first encountered by Anslow & Raistrick (1931) in their work on the isolation of 6-methylsalicylic acid. It was mentioned again by Oxford, Raistrick & Simonart (1935) in their memoir on fulvic acid, and the same authors later (1939) devised a method for separating it from the accompanying griseofulvin. To extend the account of the synthetical achievements of this biochemically remarkable mould we now give a description of our researches, as far as they have gone, into the constitution of this nitrogenous product which we propose to name '*mycelianamide*', meaning an amide found in mycelium.

Mycelianamide, m.p. 170–172° decomp., is a colourless, laevorotatory, crystalline substance of empirical formula $C_{22}H_{28}O_5N_2$. It is sparingly soluble in the usual organic solvents and is very readily split both by acids and alkalis. The following is a list of the relevant facts concerning its properties and breakdown products.

(1) The compound has no basic properties but is a very weak acid, soluble in sodium carbonate solution but not in sodium bicarbonate or baryta water. It is at least a monobasic acid, but does not titrate sharply and suffers some decomposition during the titration. It has no phenolic properties. It could not be induced to give any reaction for a $>$CO group.

(2) It gives an intense reddish brown colour with ferric chloride in ethanolic solution.

(3) After hydrolysis by hot concentrated HCl two bases were isolated as hydrochlorides, viz. ammonia and the known compound ω-amino-*p*-hydroxyacetophenone (1).

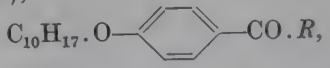
(1) 

(4) Hydrolysis by boiling dilute sulphuric acid yielded an inseparable mixture of non-volatile products. The volatile products included CO_2 (1 mol. if the hydrolysis was continued long enough), ammonia (1 mol.) and a sweet-smelling unsaturated hydrocarbon $C_{10}H_{16}$ (b.p. 177°) subsequently referred to as 'mycelene', which appears not to be identical with any known hydrocarbon of this empirical formula. Some observations concerning its constitution and relation to mycelianamide appear in a subsequent paragraph. A little acetaldehyde is also produced in this hydrolysis.

(5) Hydrolysis by boiling dilute sodium hydroxide or sodium carbonate (or even bicarbonate) yielded ammonia (approximately 1 mol.) and an optically inactive, monobasic acid $C_{17}H_{22}O_3$, m.p. 118–120°, which has no phenolic, alcoholic or ketonic properties. On further hydrolysis with dilute sulphuric acid it yielded mycelene and *p*-hydroxybenzoic acid ($C_7H_6O_3$). This decomposition can be represented very simply as follows:

$$C_{17}H_{22}O_3 = C_{10}H_{16} + C_7H_6O_3,$$

and the acid $C_{17}H_{22}O_3$ is clearly *p*-myceloxybenzoic acid (2; $R = OH$),

(2) 

the third oxygen atom possessing an ether function. It is reasonable to assume that mycelene arises from a hypothetical alcohol, mycelyl alcohol $C_{10}H_{17}OH$, by loss of water, and that the cause of the remarkable instability of the ether link in the acid $C_{17}H_{22}O_3$ must lie in the constitution of the mycelyl radical (see later).

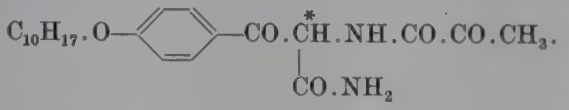
(6) Hydrolysis by cold very dilute ammonia (0·03 N), continued for several days at room temperature, yielded a neutral compound $C_{17}H_{23}O_2N$, m.p. 120°, which by further acid hydrolysis again yielded mycelene and *p*-hydroxybenzoic acid. This neutral compound is therefore p-*myceloxybenzamide* (2; $R = NH_2$). It is obviously of importance to find out if the N atom in this breakdown product arises from the ammonia used in the hydrolysis or whether it is an integral part of the molecule of mycelianamide. All attempts to obtain *p*-myceloxybenzamide from mycelianamide directly by the use of other

alkalis in dilute solution failed. These included NaOH (0·01 N and 0·03 N), methylamine, potassium carbonate, and various mixtures of potassium carbonate and sodium bicarbonate all acting in the cold at a concentration of 0·03 N. Only with methylamine was an appreciable amount of an insoluble, neutral compound obtained, and this proved to be uncrystallizable. It must be concluded that there is no evidence at the moment against the view that the N atom of *p*-myceloxybenzamide is not an integral part of the mycelianamide molecule but originates in the ammonia used in the particular mode of hydrolysis which leads to its formation.

p-Myceloxybenzamide, as obtained above, is optically inactive. Since it is almost inconceivable that racemization could occur under the particularly mild conditions of this hydrolysis the important consequence follows that the mycelyl radical does not contain an asymmetric C atom.

(7) It is an interesting fact, although not of immediate importance with respect to the structure of mycelianamide, that this mould product exerts a marked bacteriostatic action on *Staphylococcus aureus*, quantitatively almost identical with that of 4:4′-diamidino-$\alpha\gamma$-diphenoxypropane (Ashley, Barber, Ewins, Newberry & Self, 1942).

As the simplest partial structural formula which will explain the above facts we suggest (3), viz. the *amide* of O-*mycelyl*-N-*pyruvyl*-β-*keto-tyrosine*, a structure which is obviously plausible from a phytochemical point of view.

(3) 

Accepting the view previously arrived at that the $C_{10}H_{17}$ radical does not contain an asymmetric C atom it will be seen that (3) contains one asymmetric C atom, marked with an asterisk. The fact that mycelianamide is optically active eliminates one structure which is otherwise as satisfactory as (3), viz. that in which the side chain is

$$.CO.CH_2.NH.CO.CO.CH_2.CONH_2.$$

An attempt will now be made to account for the known facts listed above, concerning mycelianamide on the basis of (3).

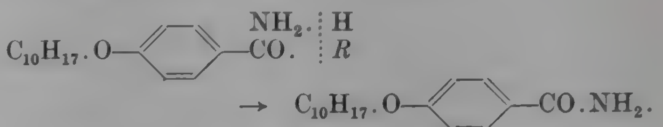
(1) (3) contains no basic N atom, nor a COOH group. It ought, however, to be weakly acidic by virtue of the grouping .CO.CH.CO., which is known to confer acidity on benzoylacetic ester and related compounds.

(2) The presence of this same grouping will explain the reddish brown colour with ferric chloride.

(3 and 4) Acid hydrolysis may be supposed to remove the mycelyl radical leaving a phenolic OH, to convert the acid amide grouping to COOH, and also to split the NH.CO link. The resulting amino derivative of hydroxybenzoylacetic acid would readily lose CO_2 on further acid hydrolysis to yield structure (1). The acetaldehyde, which is produced in very small yield, might arise from the acetyl group at the end of the side-chain in (3) by the following side-reaction:

$$R.CO.CO.CH_3+H_2O=R.COOH+CH_3.CHO.$$

(5 and 6) *p*-Myceloxybenzoic acid is formed from (3) by the ordinary alkaline breakdown of a substituted benzoylacetic acid leading to the formation of a benzoic and an acetic acid. With ammonia the following reaction must be supposed to occur:



The nearest analogy in the literature is the production of benzamide from the addition compound of ammonia and benzophenone by the action of water (Houben, 1924). The ammonia produced by the action of caustic soda on mycelianamide arises from the acid amide grouping.

A word or two may now be said on the constitution of the mycelyl radical. Four atoms of hydrogen are readily taken up by the catalytic reduction of mycelianamide in presence of palladium. Hence the mycelyl radical probably contains two double bonds, and the hypothetical mycelyl alcohol ought to be an open-chain diolefinic alcohol, in fact a near relative of geraniol. When mycelianamide was oxidized by ozone the only recognizable product was methylglyoxal (isolated as the *bis*-2:4-dinitrophenylhydrazone), which may mean that the double bonds are conjugated, but may also be interpreted to mean that mycelianamide contains the grouping $CH_3.\underset{\|}{C}.CH_2O.$, which would yield acetol (pyruvyl alcohol, $CH_3CO.CH_2OH$) by the action of ozone. The latter alcohol would be oxidized to methylglyoxal by the action of dinitrophenylhydrazine. This last explanation implies that mycelianamide is a substituted allyl ether of phenol. The allyl group in such compounds is known to be labile (Claisen, 1912) and the ready formation of mycelene by dilute acid hydrolysis may thus be explained. Mycelene has been examined by Prof. J. L. Simonsen, F.R.S., to whom we desire to express our most sincere thanks, and his observations are included in the experimental section. In brief, mycelene appears to be a monocyclic hydrocarbon containing two double bonds which are not conjugated. One of these double bonds is probably terminal, since formaldehyde is formed in quantity by the action of ozone. It seems very unlikely that mycelene is an isoprene derivative. Owing to the somewhat drastic conditions employed in the production of mycelene from mycelianamide, the constitution of the former, if known, would provide no certain clues to the constitution of the mycelyl radical in mycelianamide. It is only necessary to recall that the primary alcohol geraniol is converted into the tertiary alcohol linalol merely by heating with water, in order to render probable the supposition that mycelyl alcohol would be an extremely labile substance.

As regards the biochemical origin of mycelianamide, a possible explanation may be that *P. griseofulvum*, which, from our previous researches, is known to be an extraordinarily prolific producer of phenolic substances from glucose, also elaborates the phenolic amino-acid tyrosine far in excess of its requirements. By β-oxidation, amidation, alkylation of the OH group and acylation of the NH_2 group, the excess of tyrosine is converted into an insoluble substance, as long as the medium remains no more alkaline than sodium bicarbonate solution, and is stored in the mycelium. It is significant that mycelianamide is formed when the source of N in the medium is either NO_3^- or NH_4^+, and that the mould produces phenolic substances on both media.

EXPERIMENTAL

History of the culture used

The culture of ***Penicillium griseo-fulvum*** Dierckx used throughout this work was received in December 1929 from Prof. Ph. Biourge of the University of Louvain, and was numbered *B.* 34. It bears the London School of Hygiene Catalogue No. *P.* 38.

Extraction of mycelianamide

The cultural conditions (as regards modified Czapek-Dox medium with $NaNO_3$ as source of N) and the method of extraction of both griseofulvin and mycelianamide have already been given by Oxford *et al.* (1939). Mycelianamide is best purified by repeated crystallization from ethyl acetate in which it is moderately soluble while hot. The total yield of mycelianamide was more than twice that recorded for griseofulvin in the above memoir, i.e. about 100 g. from 2970 g. of dry mycelium derived from the growth of the mould on about 259 l. of medium containing about 21 kg. of glucose and 650 g. $NaNO_3$. Mycelianamide was also isolated from the mycelium of *P. griseo-fulvum* grown on a medium containing ammonium tartrate as source of nitrogen. The mycelium examined was obtained from our experiments in 1935 (Oxford *et al.* 1935), in which 150 flasks of the following medium were employed: glucose, 50 g.; ammonium tartrate, 5 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; $FeSO_4.7H_2O$, 0·01 g.; distilled water, 1000 ml. The sterilized flasks, each containing 350 ml. of the above medium, were inoculated with a spore suspension of *P. griseo-fulvum* and incubated at 19–21° for 50 days. The total weight of dried mycelium from three such experiments was 1119 g., yielding 81 g. of crude solid by ether extraction. By crystallization from benzene 51·7 g. of fairly pure mycelianamide m.p. 165° was obtained, containing, however, about 7% of a neutral, amorphous impurity which could not be removed by recrystallization from any solvent. About 20 g. of pure mycelianamide was eventually obtained by extraction with cold very dilute NaOH, immediate acidification of the filtrate, and repeated recrystallization of the dried precipitate. Because of the difficulty of removal of the amorphous impurity it is recommended that the mould be grown on the glucose-$NaNO_3$ medium if it is desired to prepare mycelianamide in quantity.

Analysis and general properties of mycelianamide

Mycelianamide crystallizes from ethyl acetate in colourless, shining leaflets, m.p. 170–172° with vigorous decomposition. (Found: C, 66·09, 66·12, 66·20, 66·15; H, 6·94, 7·12, 7·20, 7·07; N, 7·06, 6·69, 7·33, 7·46%; mol. wt. (cryoscopic in dioxan), 400·5. $C_{22}H_{28}O_5N_2$ requires C, 65·97; H, 7·05; N, 7·00%; mol. wt. 400.) It contains no S, Cl, OCH_3 or NCH_3.

0·1729 g. dissolved in ethanol (10 ml.) and water (3 ml.) required 7·58 ml. 0·1 N-NaOH for neutralization to phenolphthalein; hence equivalent = 228, but the end point was very indefinite and was further complicated by the production of a yellow colour after the addition of the first equivalent of alkali. Some unchanged substance could be obtained by recrystallization of the product obtained by *immediate* acidification of the titrated liquid.

Optical rotation: $[\alpha]_{5461}^{19^\circ} = -217^\circ$; $[\alpha]_{5790}^{19^\circ} = -182^\circ$ ($c =$ 0·8688 in chloroform).

It is sparingly soluble in the cold in ethanol, chloroform, ether, benzene, ethyl acetate and glacial acetic acid; readily soluble in acetone and dioxan.

Mycelianamide is unchanged by boiling water, but is very unstable to cold, dilute NaOH, the solution, which froths readily like a soap solution, soon darkening in colour and acquiring the fragrant lavender-like odour referred to earlier. It is also soluble in Na_2CO_3 solution but insoluble in dilute $NaHCO_3$, $Ba(OH)_2$ or HCl. It forms a series of insoluble *salts* and even the Na salt is partly precipitated by the addition of NaCl to its neutral aqueous solution. The insoluble ferric salt is reddish brown, and the insoluble Cu and Ni salts are green. The yellow insoluble Ag salt becomes black on standing.

Colour reactions. A deep reddish brown colour results when a few drops of very dilute $FeCl_3$ are added to a solution of mycelianamide in ethanol or glacial acetic acid. The colour is discharged by dilute HCl. With 70% H_2SO_4 the colour was first yellow, becoming orange and finally deep brownish red on standing. Mycelianamide does not give the Millon reaction, the murexide reaction, the biuret reaction, or the *p*-dimethylaminobenzaldehyde reaction for indole derivatives. It could not be induced to form a picrate or methiodide, or give any reaction for a $\rangle$CO group.

Mycelianamide is highly unsaturated and absorbs Br_2 rapidly in chloroform solution. Nothing crystalline resulted from numerous oxidation experiments with $KMnO_4$, H_2O_2, HNO_3 in glacial acetic acid and alkaline I_2. Iodoform was produced in the last instance. Attempted acetylation by acetic anhydride in pyridine solution soon yielded a very dark-coloured liquid from which only amorphous products could be obtained. Methylation by diazomethane yielded a yellow, viscid oil, with an odour of impure acetamide, which was insoluble in both acids and alkalis.

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Catalytic reduction of mycelianamide

Mycelianamide (0·832 g.) dissolved in ethyl acetate (100 ml.) was reduced by shaking in an atmosphere of H_2 with a catalyst prepared from palladium chloride (0·1 g.) and norite charcoal (1 g.). Uptake of H_2 was at first rapid (60 ml. in 17 min.) and ceased when 100 ml. had been absorbed in 146 min. (theor. for saturation of two double bonds = 93 ml.). After filtration, the solvent was removed *in vacuo* below 40° to leave a thick brown syrup which became an amorphous solid on treatment with boiling light petroleum, from which nothing crystalline could be obtained. It would be expected that new asymmetric centres might be formed by reduction and the product would then be a complex mixture containing several stereoisomers.

Oxidation of mycelianamide by ozone

Ozonized O_2 was passed through a solution of mycelianamide (0·5 g.) in chloroform (50 ml.) for 6 hr., the temperature being maintained at $-10°$ to 0°. The ozone was completely absorbed throughout and there appeared to be much unchanged substance at the end. The liquid was twice extracted with water and twice with $NaHCO_3$ solution, and the extracts, after acidification with dilute HCl, were added to excess of 2:4-dinitrophenylhydrazine in 2N-HCl. A precipitate slowly formed and was collected after several days. Fine, orange-red needles were obtained by crystallization from aqueous dioxan, m.p. 302° decomp., alone or mixed with the authentic *bis*-2:4-dinitrophenylhydrazone of methylglyoxal. The respective colour reactions with ethanolic KOH were also identical.

Action of hot conc. HCl *on mycelianamide; formation of* NH_4Cl *and the hydrochloride of ω-amino-*p*-hydroxyacetophenone* (1)

Mycelianamide (10 g.) and conc. HCl (1000 ml.; *d* 1·16) were heated together for 15 hr. at 100–105° with frequent shaking. The dark-coloured liquid was then filtered from tar, repeatedly extracted with ether until the extracts were almost colourless, freed from dissolved ether by the passage of a rapid stream of air for several hours, and finally taken to dryness *in vacuo* at 50–60°. The last traces of HCl were removed from the almost black solid residue by repeated shaking with ether. The residue was taken up in a little hot ethanol, and the undissolved part (mostly NH_4Cl) was benzoylated by the Schotten-Baumann method to yield benzamide, m.p. and mixed m.p. 126–128°. Fractional precipitation of the ethanolic extract with ether yielded a crystalline hydrochloride (prisms) and NH_4Cl. The latter, precipitated in a very finely divided condition, came through the filter on suction and was thus removed.

The crude hydrochloride remaining on the filter (1·2 g.) was purified by repeated crystallization from ethanol-ether. It formed almost colourless, compact prisms, m.p. 249–253° decomp., which were readily soluble in water to give a neutral solution which gave a purple colour with $FeCl_3$, an intense yellow colour with bleaching powder solution, a positive Millon reaction but a negative Denigè-Mörner reaction for tyrosine. The compound was the hydrochloride of a primary amine, since an effervescence was immediately observed when very dilute HCl was added to its solution in very dilute $NaNO_2$. The NH_2 was not directly attached to a benzene nucleus since no dyestuff was produced by diazotization and coupling with β-naphthol. Furthermore, the phenolic OH appeared to have a free position *ortho* or *para* to it since an intense orange-red colour was given with diazotized sulphanilic acid. The amine contained no —CHO group since it did not restore the colour to Schiff's reagent.

A *platinichloride* was prepared by addition of 5% platinic chloride solution (10 ml.) to a solution of the hydrochloride (0·15 g.) in water (2 ml.). The mixture was filtered and kept in the cold room when orange needles and prisms separated; m.p. 224° decomp. (Found, on material dried at 15°: C, 25·74; H, 3·31; N, 3·90, 4·02; Pt, 25·89%; loss at 100° *in vacuo*, 4·53%. $(C_8H_9O_2N)_2 . H_2PtCl_6 . 2H_2O$ requires C, 25·65; H, 3·23; N, 3·74; Pt, 26·08; $2H_2O$, 4·81%. Found, on material dried at 100° *in vacuo*: C, 26·94; H, 2·99; Pt, 26·66%. $(C_8H_9O_2N)_2 . H_2PtCl_6$ requires C, 26·96; H, 2·83; Pt, 27·39%.)

The amine hydrochloride also yielded a picrate (needles, m.p. 188–190° decomp.) and a benzoate, by the Schotten-Baumann method, which formed long needles from ethanol, m.p. 177–178°. (Found: C, 73·49, 73·30; H, 4·83, 4·77; N, 4·33, 4·21%; mol. wt. (cryoscopic in dioxan), 303. Calc. for $C_{22}H_{17}O_4N$ (dibenzoate of $C_8H_9O_2N$): C, 73·52; H, 4·77; N, 3·90%; mol. wt. 359.)

The free amine is therefore $C_8H_9O_2N$, and the most likely structure which will fit the facts recorded above is $HO . C_6H_4 . CO . CH_2 . NH_2$.

*ω-Amino-*p*-hydroxyacetophenone* (1) was first made synthetically by Tutin, Caton & Hann (1909) who recorded the following constants: m.p. 190–193° decomp.; hydrochloride, prisms, m.p. 245–252° decomp.; picrate, needles, m.p. 192° decomp.; dibenzoate, needles, m.p. 173–174°. The melting points given for the three derivatives are all close to the values given above for the corresponding derivatives of the amine from mycelianamide. Through the great kindness of Dr T. A. Henry we have been able to make a direct comparison of our hydrochloride with that made by Tutin *et al.* in 1909 at the Wellcome Chemical Research Laboratories. The latter hydrochloride gave a purple colour with $FeCl_3$ and an intense yellow colour with bleaching powder solution; it melted at 246–253° alone or mixed with our hydrochloride, and readily yielded a benzoate of m.p. 176–178°, alone or mixed with the benzoate derived from our hydrochloride. There can thus be no doubt that the amine derived from mycelianamide is ω-amino-*p*-hydroxyacetophenone, but, in our opinion, the description of this compound given by Tutin *et al.* (1909) requires amendment in one or two particulars. We have prepared the free amine from their specimen of the hydrochloride, and also from our own, and find that it does not melt at 190–193° as stated, but rather begins to darken at 190° and progressively darkens and softens from 200 to 300°, with production of a sublimate of yellow needles which do not melt at 310°. The amine sublimes readily at 150–170° *in vacuo* to yield a product which behaves exactly as above. (Found on sublimed material from the product obtained by the action of Na_2CO_3 on our amine hydrochloride: C, 63·68; H, 6·07; N, 9·50%. $C_8H_9O_2N$ requires C, 63·54; H, 6·00; N, 9·27%.) Tutin *et al.* (1909) further stated that the platinichloride and aurichloride were both too soluble to admit of isolation. In our experience this statement applies only to the aurichloride.

Hydrolysis with boiling dilute H_2SO_4*; production of* NH_3 *(1 mol.),* CO_2 *(1 mol.) and mycelene,* $C_{10}H_{16}$

Mycelianamide (7·85 g.) suspended in a mixture of water (800 ml.) and N-H_2SO_4 (450 ml.) was refluxed for 9·5 hr., in a stream of N_2, until the solid had completely disappeared and had been replaced by a dark-coloured oil floating on a pale-yellow liquid. CO_2 was evolved slowly but continuously throughout and was absorbed in baryta bubblers, the total amount being equivalent to 22·5 ml. of N-$Ba(OH)_2$, i.e. 0·57 mol. of CO_2/mol. of mycelianamide. In a separate experiment, in which the hydrolysis was continued until CO_2 ceased to be evolved, mycelianamide (1·0618 g.) was refluxed with 2N-H_2SO_4 (60 ml.) in a stream of N_2 for 13 hr. CO_2 was evolved equivalent to 53·8 ml. 0·1N-$Ba(OH)_2$. Theor. for 1 mol. CO_2/mol. of mycelianamide, 53·1 ml.

The reaction liquid from the 7·85 g. of mycelianamide was distilled in steam for 5 hr. until no more oil came over.

The acid hydrolysis liquid was treated as follows. A sample was made alkaline and distilled to yield 55% of the N originally present in the mycelianamide, $C_{22}H_{28}O_5N_2$, as a volatile base which was identified as NH_3 by conversion into benzamide, m.p. and mixed m.p. 126–127°. Hence only one of the two N atoms present was accounted for. Part of the remaining N was present in an intractable tar (wt. 0·5 g.) which was formed during the hydrolysis. (Hydrolysis of mycelianamide with 30% (w/w) H_2SO_4 yielded much the same results, save that the amount of tar formed was much greater, and 52% of the N present in the mycelianamide was accounted for as NH_3.) All attempts to isolate crystalline compounds in a state of purity from the hydrolysis liquid failed so that the fate of the second N atom present in mycelianamide remains undetermined. At least two crystalline compounds appeared to be present, viz. clusters of massive plates, m.p. about 150°, and small feathery crystals, m.p. about 100°, both soluble in ether and crystallizing from benzene. Neither substance could be obtained free from adhering oil in quantities sufficient for analysis and characterization. Neither of them was identical with *p*-myceloxybenzoic acid, $C_{17}H_{22}O_3$, m.p. 118–120°, formed in the alkaline hydrolysis (see p. 328).

The steam distillate was treated as follows. A sample was titrated but no volatile acid was detected. The oil was extracted with ether, and the extract dried over anhydrous Na_2SO_4. After removal of the solvent the residual oil was distilled at atmospheric pressure to yield 1·56 g. of distillate, the middle fraction of which (0·68 g.) came over at 177–178° and was almost colourless. By redistillation *in vacuo* it was obtained as a colourless limpid oil with a strong lavender-like odour. (Found: C, 84·26, 84·51; H, 11·21, 11·07; O (by difference), 4·53, 4·42%.) The atomic C : H ratio calculated from the above figures is 1 : 1·57, and since the boiling point is close to that of several terpenes, e.g. *d*-limonene, $C_{10}H_{16}$, boils at 176°, there can be little doubt that the product is essentially a hydrocarbon. The oxygen-containing impurity was not water since the oil submitted to analysis gave an absolutely clear and bright solution in light petroleum.

Several grams of the once-distilled hydrocarbon were prepared and were examined by Prof. J. L. Simonsen, F.R.S., at that time of the University College of North Wales, Bangor, who in a letter dated 24 April 1937, reported as follows.

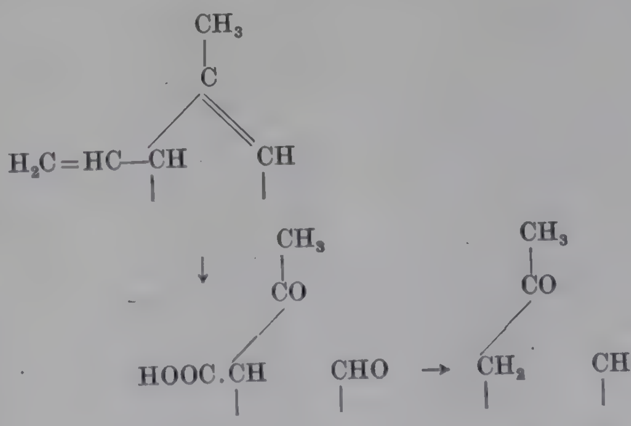
‘The hydrocarbon had b.p. 73–75°/20 mm., $d_{25°}^{25°}$ 0·8583, $n_D^{20°}$ 1·4780, $[\alpha]_{5461}=0°$. Molecular refractivity, 45·1; calc. $C_{10}H_{16}$ with two ethylenic linkages 44·6. (Found on sample distilled over sodium: C, 84·6; H, 11·4; after 3 days C, 83·5; H, 11·1; after 4 days C, 83·7; H, 11·0%.) The hydrocarbon is obviously very readily oxidized. It polymerizes to a gum on standing.

The hydrocarbon (3·5 g.) in ethyl acetate (30 ml.) was ozonized at 0°; a considerable quantity of formaldehyde was present in the issuing gases (dimedone test). After removal of the ethyl acetate *in vacuo*, the ozonide was decomposed in water (10 ml.) by heating on the water bath for 1 hr. The oil was extracted with ether, the ethereal extract washed with Na_2CO_3 solution and NaOH, dried over $MgSO_4$ and the solvent removed. A pleasant smelling oil (2 g.) remained. This gave no colour with $FeCl_3$, reduced Fehling's solution and gave a silver mirror (poor). Satisfactory products were not obtained with semicarbazide, phenylsemicarbazide, *p*-nitrophenylhydrazone. The 3:5-dinitrophenylhydrazone separated at once as an amorphous orange-yellow powder. It was very sparingly soluble in ethanol, but crystallized from ethyl acetate-ethanol in what appeared to be rosettes of terracotta prisms, m.p. 170–173° raised to 180–182° by recrystallization. (Found: C, 48·1; H, 4·4; N, 22·1. $C_{20}H_{22}O_8N_8$, i.e. *bis*-dinitrophenylhydrazone of $C_8H_{14}O_2$, requires C, 47·8; H, 4·3; N, 22·3%.)

The acid fractions weighed respectively 0·3 and 0·8 g. Both gave amorphous derivatives with carbonyl reagents, and bromoform with NaOBr. They were combined and oxidized in alkaline solution with this reagent. The resulting acid was a viscid oil. It did not give an anhydride with acetyl chloride; the *p*-bromophenacyl derivative was a viscid gum.

I would suggest that the following conclusions can be drawn tentatively:

(1) The hydrocarbon is cyclic and contains two ethylenic linkages; one of these is exocyclic (formaldehyde) and the other as the group Me.C:CH—. The formation of a keto-aldehyde having the composition $C_8H_{14}O_2$ might result in accordance with the following scheme:



(2) This receives support from the fact that the acid fraction does contain the grouping MeCO— as shown by the NaOBr oxidation with the formation of bromoform. The non-formation of an anhydride suggests that the dibasic acid (if it be such) is an adipic acid.

(3) It seems very unlikely that the hydrocarbon is an isoprene derivative.’

Hydrolysis with moderately strong H_2SO_4*; production of acetaldehyde*

A mixture of mycelianamide (2 g.) and 30% (w/w) H_2SO_4 (200 ml.) was boiled in a flask connected to a long condenser, the liquid being replaced by distilled water, from a dropping funnel, as fast as it distilled over. After 7 hr. the distillate (900 ml.) was filtered free from oil and redistilled, the first 100 ml. being collected in an ice-cooled receiver. To this was added conc. HCl (10 ml.) and a saturated solution of 2:4-dinitrophenylhydrazine in 2N-HCl (25 ml.), and the resulting mixture kept overnight at 0°. The precipitated dinitrophenylhydrazone, after two crystallizations from light petroleum, formed shining orange plates, m.p. 161–163°, alone or mixed with the authentic 2:4-dinitrophenylhydrazone of acetaldehyde. A mixed melting point with formaldehyde 2:4-dinitrophenylhydrazone showed a large depression. The yield of acetaldehyde in the above hydrolysis was very small.

Hydrolysis with boiling dilute NaOH, Na_2CO_3 *or* $NaHCO_3$*; formation of* NH_3 *and* p-*myceloxybenzoic acid* (2; $R = OH$)

Similar results were obtained with all three alkalis save that the liberation of NH_3 was very slow with $NaHCO_3$ as the hydrolyzing agent. The following is a typical experiment with Na_2CO_3. A solution of mycelianamide (1 g.) in 0·5N-Na_2CO_3 (100 ml.) was very slowly distilled in a current of N_2. After about 3 hr. the distillate no longer turned red litmus blue and the total volatile base which had distilled over was equivalent to 21·8 ml. of 0·1N-HCl, i.e. contained 44% of the N originally present in 1 g. of mycelianamide. The HCl-neutralized distillate, which contained a few drops of mycelene, was taken to dryness *in vacuo* to yield NH_4Cl (fern-like aggregates of needles, m.p. above 300°) which, when benzoylated by the Schotten-Baumann method, yielded benzamide, m.p. and mixed m.p. 126–128°. The brownish red liquid remaining in the distillation flask was acidified with dilute H_2SO_4 and exhaustively extracted with ether to yield a dark-brown syrup, a very small portion of which was soluble in hot light petroleum. Concentration of the petroleum extract yielded colourless slender needles (40 mg.), m.p. 118–120°, after purification by recrystallization from light petroleum. (Found: C, 74·20, 74·39; H, 8·10, 8·10%; N, nil.; mol. wt. (cryoscopic in camphor), 276, 280. $C_{17}H_{22}O_3$ requires C, 74·39; H, 8·09%; mol. wt. 274.)

The product (0·0548 g.) in a mixture of ethanol (6 ml.) and water (4 ml.) required 2·03 ml. of 0·1N-NaOH for neutralization to phenolphthalein; hence equivalent = 270 and the acid is monobasic. The titrated liquid was optically inactive and the unchanged acid was recovered from it by acidification.

The acid $C_{17}H_{22}O_3$ (*p-myceloxybenzoic acid*) is insoluble in water but readily soluble in all organic solvents with the exception of light petroleum. It forms a sparingly soluble Na salt, gives no colour with $FeCl_3$ and only a faint Millon reaction on warming. It could not be induced to form a semicarbazone or 2:4-dinitrophenylhydrazone, and was recovered unchanged after treatment with acetic anhydride in pyridine at room temperature for 2 days.

Hydrolysis of the acid $C_{17}H_{22}O_3$ *with production of mycelene and* p-*hydroxybenzoic acid*

The acid (0·6 g.) was treated for 3 hr. with boiling 0·5N-H_2SO_4 (240 ml.) under reflux condenser. One hundred ml. were then distilled off to yield discrete drops of oil in the first runnings, with the characteristic odour of mycelene. The liquid in the distillation flask was cooled, extracted four times with ether, and the extracts dried over anhydrous Na_2SO_4. The yellow crystalline residue (0·3 g.) left after removal of the solvent was crystallized from benzene containing some chloroform to yield colourless flat prisms, m.p. 210–212°, alone or mixed with authentic *p*-hydroxybenzoic acid, m.p. 212°. (Found: C, 61·02, 60·87; H, 4·30, 4·38%. Calc. for $C_7H_6O_3$: C, 60·84; H, 4·38%.)

Action of cold dilute ammonia on mycelianamide; production of p-*myceloxybenzamide* (2; $R = NH_2$)

Mycelianamide (50 mg.) was dissolved in N-NH_4OH (6·6 ml.) by gentle shaking, and the solution *immediately* diluted to 200 ml. with distilled water. A crystalline deposit began to appear after standing for 1 day at room temperature and was collected on the fourth day. The filtrate deposited very little more of the product after standing for a further 3 days; total yield, 15 mg. of m.p. about 120°. p-*Myceloxybenzamide* formed colourless silky needles from benzene containing some light petroleum; m.p. 120°, considerably depressed on admixture with *p*-myceloxybenzoic acid, m.p. 118–120°. (Found: C, 74·65, 74·8; H, 8·43, 8·56; N, 5·0, 5·2%. $C_{17}H_{23}O_2N$ requires C, 74·66; H, 8·49; N, 5·1%.) A 1·1% solution in acetone gave absolutely no rotation in a 0·5 dm. tube.

Hydrolysis. The above compound, m.p. 120° (90 mg.), was treated with boiling 20% (w/w) H_2SO_4 (25 ml.) under reflux condenser for 3 hr. Drops of volatile oil were observed in the condenser after a very short time and the fragrant odour was in evidence (formation of mycelene). The reaction liquid was cooled, filtered, extracted once with a little light petroleum and then twice with ether. An almost colourless solid was obtained from the ether extract, which, after treatment with a little boiling benzene and boiling carbon tetrachloride, melted at 210–212°. Mixed with authentic *p*-hydroxybenzoic acid the m.p. was 212–214°. The colour reactions with Millon's reagent and with $FeCl_3$ were identical with those given by *p*-hydroxybenzoic acid.

Bacteriostatic action of mycelianamide

The method of testing used was that described by Oxford (1942) modified to meet the case of a compound insoluble in water but soluble in ammonia. A 1 : 4000 solution of mycelianamide in 0·03N-NH_4OH was made as described above, sterile N-NH_4OH and sterile distilled water being used, and employed *immediately* for the test. Volumes up to 1 ml. of this solution were added aseptically to 4 ml. quantities of sterile 2% glucose broth and the volumes in the tubes made up to 5 ml., if necessary, by the addition of sterile 0·03N-NH_4OH. The controls contained 4 ml. of sterile glucose broth plus 1 ml. of sterile 0·03N-NH_4OH. The inoculum given in all cases was 0·04 ml. of a 1-day culture in glucose broth which had been diluted 1000 times. The tubes were read after 1 day's incubation at 37°. All the controls grew normally. The results are summarized in

Table 1. *Limiting dilutions of mycelianamide for complete inhibition of growth of various bacteria*

1 : 20,000 inhibits	1 : 33,000 inhibits	1 : 50,000 inhibits
Staph. aureus strains* 3093, 3095, 3750 and 3761	*Staph. albus* 3256*	*B. anthracis* 5444*
Strep. viridans 3166*	*Staph. aureus* 4163*	
Strep. pyogenes 2432* (on plain broth)		

* Of the National Collection of Type Cultures.

Table 1, from which it will be seen that *Staphylococcus aureus* and other Gram positive bacteria are completely inhibited by mycelianamide at concentrations of 0·002–0·005%. The compound has no marked action on Gram negative bacteria.

Mycelianamide was equally effective against Gram positive bacteria on plain heart broth containing no added glucose, and almost equally effective if an undiluted culture was used for sowing the tubes. Through the courtesy of Dr A. J. Ewins, F.R.S., we were able to compare the bacteriostatic action of mycelianamide with that of certain aromatic diamidines of comparable molecular complexity, and have found that 4:4′-diamidino-$\alpha\gamma$-diphenoxypropane ($C_{17}H_{20}O_2N_4$; mol. wt. = 312) is almost identically potent when tested in dilute aqueous solution, in that 1 part in 40,000 would inhibit the growth of *Staph. aureus* No. 3095, and 1 part in 20,000 would inhibit the growth of *Staph. aureus* No. 3750 for 1 day.

SUMMARY

1. A hitherto undescribed, colourless, crystalline, metabolic product, *mycelianamide*, $C_{22}H_{28}O_5N_2$, m.p. 170–172° decomp., has been isolated from the mycelium of *Penicillium griseo-fulvum* Dierckx.

2. Acid hydrolysis of mycelianamide under different conditions gives a new hydrocarbon, *mycelene*, $C_{10}H_{16}$, ω-amino-*p*-hydroxyacetophenone, acetaldehyde, ammonia and carbon dioxide.

3. Alkaline hydrolysis gives ammonia and a monobasic acid, $C_{17}H_{22}O_3$, which on acid hydrolysis affords mycelene and *p*-hydroxybenzoic acid.

4. Consideration of these and other reactions leads to the tentative conclusion that mycelianamide may be the *amide* of *O-mycelyl-N-pyruvyl-β-keto-tyrosine*.

5. Mycelianamide completely inhibits the growth of a number of Gram positive bacteria *in vitro* at concentrations of 1 : 20,000 to 1 : 50,000, but has no marked action on Gram negative bacteria.

We desire to thank Prof. J. L. Simonsen, F.R.S., for his examination of the hydrocarbon, mycelene; Dr A. J. Ewins, F.R.S., for a gift of aromatic diamidines; and Dr T. A. Henry for a gift of ω-amino-*p*-hydroxyacetophenone.

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Biochemistry of the Wood-rotting Fungi

5. THE PRODUCTION OF D-THREITOL (*l*-ERYTHRITOL) BY *ARMILLARIA MELLEA* (VAHL) QUÉLET

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The tetrahydric alcohol until recently known as erythritol is capable of existing in three stereoisomeric forms (if we exclude external compensation), the optical relationships being the same as for tartaric acid. The optically inactive internally compensated form, known as *meso*erythritol or *i*-erythritol has frequently been observed in nature. It is found in the algae *Protococcus vulgaris* (Lamy, 1852) and *Trentepohlia Jolithus* (Bamberger & Landsiedl, 1900). It also occurs in lichens of the genus *Roccella* (Stenhouse, 1848; Hesse, 1904) usually in the form of erythrin from which it can be obtained in the free state by hydrolysis. It has also been isolated from various fungi. Zellner (1910) obtained it from spores of *Ustilago maydis*, Oxford & Raistrick (1935) from the mycelium of *Penicillium brevi-*

compactum Dierckx and of *P. cyclopium* Westling, and Stodola (1946) from *Aspergillus terreus* Thom.

There is, however, no record in the literature of the natural occurrence of the optically active forms of erythritol, although they have been prepared by synthetic methods by Maquenne (1900) and others. This gap has now been partially filled by the isolation from a natural source of the form of erythritol which is dextrorotatory in water and laevorotatory in ethanol and which in accordance with the modern nomenclature is described as D-threitol. It was obtained in a yield of about 13 % on dry weight from the mycelial tissue of a freshly isolated laboratory culture of *Armillaria mellea* (Vahl) Quélet, one of the higher fungi. When subcultured in the laboratory under our conditions, the fungus rapidly lost its power of producing D-threitol. The species is widely distributed and has been reported as a cause of damage to a variety of plants ranging from forest trees to shrubs and herbaceous plants. It is very common on tree stumps, but may become parasitic, attacking the base of living trees.

The new product is apparently identical with the substance synthesized by Maquenne from natural xylose and described under the name of '*l*-erythritol'. The name was later changed to '*d*-erythritol' in accordance with the Rosanoff convention for sugars, but recently it has been thought advisable to change the name to D-threitol to relate it more closely to the tetrose threose.

EXPERIMENTAL

Culture. The culture was obtained by transplantation on to malt agar of a fragment of tissue from the fruiting body of *Armillaria mellea* found growing in a large clump at the foot of a horse-chestnut tree at Pinner, Middlesex. The inoculum was taken under sterile conditions from a newly exposed surface obtained by breaking the pileus.

Medium and conditions of culture. Two culture solutions were prepared from a basal medium of the following composition: glucose 30 g.; KH_2PO_4 2·5 g.; $MgSO_4.7H_2O$ 1·0 g.; $FeSO_4.7H_2O$ 0·01 g.; $ZnSO_4.7H_2O$ 0·01 g.; $MnSO_4.4H_2O$ 0·005 g.; $CuSO_4.5H_2O$ 0·005 g.; $Na_2B_4O_7.10H_2O$ 0·005 g.; NH_4Cl 2·5 g.; water 1000 ml. For culture medium *A* an addition of 10 g. of corn-steep powder, and for medium *B* an addition of 0·1 g. of Marmite was made.

The medium was distributed in conical flasks of 1 l. capacity, 350 ml. to each flask. It was sterilized by autoclaving at 110° and inoculated with fragments of tissue from slopes on malt agar containing an addition of 1% of corn-steep solids. In culture on liquid media of depth about 1 in. the fungus forms a mass of tangled rhizomorphs, the lower portion of the mass eventually coming to rest on the bottom of the vessel and the upper portion appearing above the surface of the liquid.

Growth and harvesting of fungus. The rate of growth and utilization of sugar is much more rapid on medium *A* (corn steep) than on medium *B* (Marmite). The contents of single flasks were harvested after varying intervals of time. The culture solution was filtered from the total mycelium obtained from the six single flasks (Table 1). The mycelium was dried *in vacuo* (dry weight 20·00 g.), powdered and subjected to continuous extraction. After a preliminary extraction with light petroleum to remove lipid material the solid was subjected to exhaustive extraction with dry ether (16 working days). Even after this length of time, matter was still being extracted, but the daily yield had fallen to less than 0·1 g. of extractive. During the extraction a crystalline substance separated on the walls of the extraction flask and was scraped off daily before renewing the extraction. Evaporation of the ether gave a little more of this crystalline material (0·12 g.) and 0·25 g. of a non-crystalline oil. The extraction was continued with ethyl acetate, but only a further 0·05 g. of the crystalline solid was obtained; the total weight of crystals extracted was 2·62 g. or 13 % of the dry weight of mycelium.

Table 1. *Rate of growth and utilization of glucose by* Armillaria mellea *on synthetic media with added corn-steep solids* (*A*) or *Marmite* (*B*)

	Dry mycelium (g./flask)		Residual glucose by polarimeter (%)	
Medium	*A*	*B*	*A*	*B*
Period of growth (days)				
11	3·02	—	2·05	—
24	4·82	1·32	0·09	2·78
35	6·82	1·79	0·00	2·28
45	—	2·23	—	2·11

Identification of the crystalline solid as D-*threitol.* The crystalline solid was recrystallized from absolute ethanol and was thus obtained in the form of long colourless silky needles with a sweet taste. The m.p. was 90–91° (corr.), not changed by further recrystallization. The product dissolved readily in water to give a neutral solution, and gave a strong reaction for primary or secondary hydroxyl groups in the test of Fearon & Mitchell (1932). (Found: C, 39·4, 39·5; H, 8·3, 8·3. Calc. for $C_4H_{10}O_4$: C, 39·4; H, 8·3 %.) Tests for N, S, and halogen were negative. The substance was found to be optically active; the following rotations were observed. In 90 % ethanol, $[\alpha]_{5780}^{20^\circ} = -13\cdot3^\circ$, $[\alpha]_{5461}^{20^\circ} = -14\cdot5^\circ$ ($c = 5\%$). In water, $[\alpha]_{5780}^{20^\circ} = +4\cdot7^\circ$, $[\alpha]_{5461}^{20^\circ} = +5\cdot5^\circ$ ($c = 5\%$).

Dibenzylidene derivative. When the substance (0·1 g.) was treated with conc. HCl (2 ml.) and benzaldehyde (0·2 ml.) a colourless crystalline solid, insoluble in water, was obtained. This was collected, washed, dried and recrystallized from ethanol, then from benzene, and was thus obtained in the form of needles of m.p. 215–222° (corr.). (Found: C, 72·2; H, 6·2. Calc. for $C_{18}H_{18}O_4$: C, 72·5; H, 6·1 %.)

The properties of the fungal product are thus in general agreement with those described by Maquenne (1900) for '*l*-erythritol' now known as D-threitol. Complete identity of the product with D-threitol was established by the synthesis of the latter substance.

Preparation of D-*threitol.* The method chosen was that of Hockett (1935) who prepared dibenzylidene-D-threitol via D-threose from D-xylose. The dibenzylidene compound was hydrolyzed in aqueous H_2SO_4 to yield D-threitol.

Tetra-acetyl-D-xylononitrile. Early experiments gave very low yields even when the reactants were vigorously dried. The following reacetylation procedure increased the yield from about 20 to 52 % of theoretical (Hockett (1935) claims 48 %).

After the acetic anhydride had all been added the mixture was held at 70–75° for 15 min. and evaporated at this temperature under reduced pressure. When most of the solvent was removed a volume of acetic anhydride equal to half that originally employed was added. After 15 min. at 70–75° the solvent was evaporated and the procedure repeated with fresh acetic anhydride. After the final evaporation water was added and the mixture was boiled for 0·5 min. to decompose acetic anhydride. On cooling, the product solidified. It was collected, washed, dried and recrystallized from ethanol. It had m.p. 82–83° and was coloured slightly brown, but was sufficiently pure for the next stage.

D-Threose diacetamide. As the purification method of Hockett gave only a low yield it was decided to dispense with purification at this stage. The crude product, after evaporation of NH_3, partly crystallized on standing.

Dibenzylidene-D-threitol. The crude D-threose diacetamide was hydrolyzed in 0·1 N-H_2SO_4 at 100° for 6 hr. the solution being maintained acid to congo red by addition of 2 N-H_2SO_4 as required. The deep red solution was reduced with Na amalgam. Complete reduction (as indicated by Fehling's test) could not be effected, but when 80–90 % reduction was attained the solution was adjusted to pH 6 and evaporated to small bulk. Ethanol was added and the mixture was cooled and filtered. The filtrate was evaporated to dryness, and the residue was taken up in conc. HCl (10 vol. based on the theoretical content of D-threitol) and shaken with benzaldehyde (2 vol. on same basis); the dibenzylidene derivative separated at once. The mixture was poured into ice and ether, warmed to 0° and the product collected, washed and dried. Yields were very variable and never greater than 23 % of theoretical, based on nitrile, the best yields being obtained with small scale preparations using up to 12 g. of the nitrile. Recrystallized from benzene the product had m.p. 217–222° (corr.).

D-Threitol. The dibenzylidene compound (3·5 g.) was added to 2 N-H_2SO_4 (100 ml.), the mixture boiled until solution was complete and the benzaldehyde distilled off in steam. A slight excess of $CaCO_3$ was added, and the mixture was filtered. The filtrate was adjusted to pH 6 with H_2SO_4 and evaporated to dryness. The D-threitol was extracted from the solid residue by boiling with ethanol. The ethanol was removed and the residue crystallized from *n*-butanol. Yield 0·94 g. (66 %), m.p. 90–91° (corr.). A further 10 % was obtained from the mother liquors. The melting points and specific rotations of the natural and synthetic D-threitol and of their benzylidene derivatives are shown in Table 2.

Table 2. *Comparison of natural and synthetic D-threitol*

	D-Threitol							Dibenzylidene-D-threitol			
		Optical rotation							Optical rotation in chloroform ($c=1\%$)		
		In water ($c=5\%$)			In 90 % ethanol ($c=5\%$)						
	Melting point	$[\alpha]_{5461}^{20^\circ}$	$[\alpha]_{5780}^{20^\circ}$	$[\alpha]_{D}^{20^\circ}$	$[\alpha]_{5461}^{20^\circ}$	$[\alpha]_{5780}^{20^\circ}$	$[\alpha]_{D}^{20^\circ}$	Melting point	$[\alpha]_{5461}^{20^\circ}$	$[\alpha]_{5780}^{20^\circ}$	$[\alpha]_{D}^{20^\circ}$
Natural	90–91° (corr.)	+5·5	+4·7	—	−14·5	−13·3	—	215–222° (corr.)	−97	−84	—
Synthetic (our observation)	90–91° (corr.)	+5·5	+4·5	—	−14·3	−13·0	—	217–222° (corr.)	−96	−87	—
Mixture of above	90–91° (corr.)	—	—	—	—	—	—	217–220° (corr.)	—	—	—
Synthetic (Maquenne, 1900)	88°	—	—	+4·33 ($c=6\%$)	—	—	−10·50	231°	—	—	—
Synthetic (Hockett, 1935)	—	—	—	—	—	—	—	218–222°	—	—	−77·9
	—	—	—	—	—	—	—	220–222° (corr.)	—	—	−78·2

Rotation figures liable to an error of ± 5 %.

The figures for the melting point of the dibenzylidene compound of threitol quoted in the literature show a considerable range: 231° (Maquenne, 1900), 204–205° (Ruff, 1901), 218–222°, 220–222° (Hockett, 1935). Since the preparation of the compound introduces two new asymmetric carbon atoms into the molecule it is probable that the substances as obtained after recrystallization are mixtures of different stereoisomers. Melting points of dibenzylidene derivatives of other polyhydric alcohols quoted in the literature also show wide variations. The only reference found to this possibility, however, is in a paper by Radulescu & Tanasescu (1924) where two forms of *bis-o*-nitrobenzylidene-erythritol and two of *bis-m*-nitrobenzylidene-erythritol are described.

SUMMARY

A freshly isolated culture of the wood-rotting fungus *Armillaria mellea* (Vahl) Quélet, grown on a liquid culture medium, gave rise to D-threitol in the mycelium, the yield being about 13 % of dry weight.

We have to thank Sir Norman Haworth, F.R.S., for valuable advice on the nomenclature of the polyhydric alcohols. One of us (C.E.S.) is indebted to the Therapeutic Research Corporation of Great Britain Ltd. for a grant which has enabled him to take part in this work.

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An Alkali-producing Mechanism in Macerated Leaves

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While investigating the enzymic demethylation of pectin in macerated leaves, a phenomenon not previously described was observed in some members of the Cucurbitaceae (Holden, 1945). When the fibre residue, obtained by squeezing out the sap from minced leaves, was washed with distilled water, it was found that successive washes had higher pH values until a maximum of pH 9·4–9·6 was reached, and this value was maintained on continued washing. The present paper concerns further investigations of this spontaneous rise of pH. The phenomenon would appear to be of importance in the extraction of normal and virus proteins from green leaves since the infectivity of a number of plant viruses is decreased by pH values greater than 9.

MATERIAL AND METHODS

The plants examined were obtained from various sources. All the Cucurbitaceae (except marrow, cucumber and bryony) were from the University Botanic Garden, Cambridge. *Nicotiana tabacum* and *N. glutinosa* were glasshouse grown and the rest of the plants were from gardens or were found growing wild in the neighbourhood of Rothamsted.

The leaves were minced with a domestic meat mincer and the sap squeezed out by hand through madapollam. The fibre was washed by suspending in distilled water (about 5 ml./g. fibre wet weight) and again squeezing out. Measurements of pH were made with a glass electrode.

Calcium was determined on ashed material. Ashing was done in porcelain crucibles over a Bunsen burner at *c.* 500° on weighed portions of fibre or measured volumes of sap that had been dried at 100°. The dried material was moistened with a few drops of HNO_3 to assist ashing. After weighing, the ash was dissolved in N-HCl, filtered, and calcium precipitated as oxalate; the latter was dissolved in H_2SO_4 and titrated with $KMnO_4$ (0·05 N or 0·01 N depending on the amount of oxalate).

Carbonate determinations were made by the method of Hutchinson & MacLennan as described by Piper (1942) except that $Ba(OH)_2$ was used instead of NaOH to absorb the CO_2 liberated.

Phosphorus was determined colorimetrically by a modification of the method of Kuttner & Lichtenstein (1932). Values for inorganic phosphorus were obtained by developing the colour in samples without previous incineration.

EXPERIMENTAL AND RESULTS

Plants showing alkaline drift

Plants of different families, including ten genera of the Cucurbitaceae, were examined and are listed in Tables 1 and 2. As the pH of the expressed sap from the leaves in which the pH rise was first observed was neutral or slightly alkaline, in contrast to the somewhat acid saps found in most plants, other plants known to have more alkaline sap were examined. Haas (1920) had recorded a pH as high as 8·5 for the expressed sap of sweet clover leaves. These were not available, but the sap of common yellow melilot (*Melilotus altissima*), which is closely related, had a pH of 7·8. On washing the fibre, however, the pH fell to 6·3. The pH of sunflower-leaf sap was given by Gustafson (1924) as between 6·3 and 6·9. This was confirmed and both sunflower (*Helianthus annuus*) and Jerusalem artichoke (*H. tuberosus*) were found to show the pH rise, though other members of the Compositae that were examined did not. All other members of the Cucurbitaceae as well as comfrey (*Symphytum officinale*) of the Boraginaceae and stinging nettle (*Urtica dioica*) of the Urticaceae showed the phenomenon. Although the pH tended to be higher in sap from leaves which showed the drift than from those which did not, this was not always so as can be seen from Tables 1 and 2. Young leaves either did not show the pH rise at all or only to a much less marked extent.

Fig leaves (*Ficus carica*) were anomalous in that the pH rose to 8 but no higher; these cannot therefore be included in either group.

Table 1. *Calcium distribution in plants not showing alkaline drift*

Family	Common name	Latin name	pH of sap	Dry wt. as % wet wt.	Ca as % dry wt. of sap	Ca as % dry wt. of fibre	% total Ca in fibre
Solanaceae	Tobacco	*Nicotiana tabacum*	6·05	8·7	1·5	1·6	52·5
		Nicotiana glutinosa	5·28	8·9	8·2	1·8	16·8
	Tomato	*Lycopersicum esculentum*	6·20	10·6	3·7	1·9	35·0
Leguminosae	Melilot	*Melilotus altissima*	7·79	20·2	1·9	2·3	61·2
Cruciferae	Broccoli	*Brassica oleracea*	5·80	17·3	3·9	1·7	44·3
Labiatae	Dead-nettle	*Lamium purpureum*	6·11	16·3	1·4	0·9	48·7
Rosaceae	Strawberry	*Fragaria vesca*	—	32·9	0·5	1·3	82·9
Compositae	Marigold	*Calendula officinalis*	5·93	7·3	0·9	0·9	19·6
	Groundsel	*Senecio vulgaris*	5·86	8·0	1·4	0·8	38·6
	Chrysanthemum	*Chrys. hortorum*	5·80	19·6	2·5	1·4	47·3
Labiatae	Peppermint	*Mentha piperata*	6·30	14·5	5·3	1·0	44·5
Caprifoliaceae	Elder	*Sambucus nigra*	5·85	19·7	0·9	2·3	72·0

Table 2. *Calcium distribution in plants showing alkaline drift*

Family	Common name	Latin name	pH of sap	Dry wt. as % wet wt.	Ca as % dry wt. of sap	Ca as % dry wt. of fibre	% total Ca in fibre
Cucurbitaceae	Marrow	*Cucurbita ovifera*	7·00	16·1	1·7	10·2	90·3
	Cucumber	*Cucumis sativus*	7·43	11·7	4·7	11·3	85·2
		Sicyos angulata	6·29	7·6	4·2	5·3	50·0
	Calabash	*Lagenaria leucantha* v. *longissima*	6·15	11·8	6·3	4·2	38·0
		Momordica charantica	6·61	24·1	0·7	7·5	90·5
	Squirting cucumber	*Ecballium elaterium*	6·80	11·1	2·5	6·9	66·1
	Loofah	*Luffa cylindrica*	5·50	19·7	1·8	6·1	77·8
	Bryony	*Bryonia dioica*	6·98	11·1	1·1	5·9	91·3
		Thladiantha dubia	—	13·9	—	6·1	—
		Cyclanthera explodens	—	12·0	—	5·9	—
Compositae	Sunflower	*Helianthus annuus*	6·60	c. 15·0	2·3	7·3	85·3
	Jerusalem artichoke	*Helianthus tuberosus*	6·11	20·3	1·5	6·2	85·5
Urticaceae	Nettle	*Urtica dioica*	—	21·8	3·0	6·5	90·0
Boraginaceae	Comfrey	*Symphytum officinale*	7·00	13·2	0·5	3·6	96·0

The leaves of all species showing the alkaline pH drift have a characteristic dry and rough feel due to the covering of large hairs which are a conspicuous feature of all of them. A microscopical examination of sections stained in ethanolic purpurin to distinguish calcified areas (Baecker, 1930) showed that both uni- and multicellular hairs and the walls of cells in the region of the hairs were calcified. In addition cystoliths occurred beneath the upper epidermis in *Urtica dioica*.

General observations on the pH *drift*

Water volume. When the maximum pH value is reached, the volume of water used for washing does not influence the pH to any great extent. In one experiment, a wash with 10 ml. water/g. wet weight nettle fibre had a pH of 9·5 while a wash with 200 ml./g. had a pH of 9·6. No volatile alkali was found in alkaline washes, but an appreciable amount of calcium (0·15–0·35 mg./ml.), traces of phosphorus (2–4 μg.) and magnesium were present.

Milling. When minced fibre is finely ground in a triple-roller mill (Bawden & Pirie, 1944) the pH is raised even without previous washing. In some species, a pH of 9 or higher is not reached by washing minced fibre but only after grinding. The pH of fibre of leaves which do not show the high pH is raised by about 0·2 unit on milling.

Heating. The pH rise is not prevented by boiling a suspension of fibre; it may actually be speeded up and the maximum pH may be higher. Whole leaves dipped into boiling water gave an extract with a pH between 8 and 9. The action appears therefore not to be enzymic.

Effect of KCl *and* $CaCl_2$. Minced fibre washed with N-KCl solution instead of water gave the alkaline pH drift, but the soaking of fibre in 0·2 M-$CaCl_2$ solution tended to prevent the pH rise.

Alkali content of fibre. It was found that addition of dilute acid to fibre caused effervescence and evolution of CO_2. When 0·2 N-HCl was added to a minced fibre suspension to lower the pH to 3·5, the pH rose after a short time to between 7 and 8 and it

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only remained stable at 3·5 after several additions of acid. The pH did not rise above 7 between additions of acid unless the solution was squeezed out from the fibre and the fibre resuspended in water, when the pH rose to above 9. Milled fibre took up a large amount of acid in one addition and no pH rise occurred.

Calcium was found in the acid extract and quantitative determinations showed that it was present in an amount sufficient to account for most of the acid added. The calcium content of the sap washes and of the fibre was determined in the various species and the results expressed on a dry matter basis. The results given in Tables 1 and 2 show that the 'alkaline drift' plants have a much higher calcium content than those of the other group. The calcium content of the sap as a percentage of dry matter is similar in both groups, but the fibre calcium is considerably higher in plants giving the pH rise; the proportion of the total calcium which is insoluble is thus greatly increased.

The calcium content of the fibre of young leaves of 'alkaline drift' plants is lower than that of fully grown leaves. Young bryony leaves (picked in June) had a fibre calcium of only 2·2 % dry matter whereas the larger older leaves from the same plants had a value of 6·2 % dry matter.

The carbonate content of milled marrow fibre was found to account for 80 % of the total calcium content. The greater part of the calcium is thus present as $CaCO_3$.

Phosphorus content of fibre. Phosphorus determinations were made on fibres of a number of species. The P content of 'alkaline drift' fibres was considerably higher than that of fibres which do not show the drift. Table 3 gives a comparison of the P distribution in fibre of nettle and tobacco, as examples of both types of fibre.

Portions of fibre (0·5 g.) were extracted with 10 ml. 0·2 N-HCl, centrifuged after 2 hr. and a further 5 ml. acid added. After the second portion of acid had been decanted, 10 ml. ethanol-ether (7 : 3) were added to remove lipid P. After a further 2 hr., a second extraction with a 5 ml. portion of ethanol-ether followed and the fibre was then dried. (HCl (0·2 N) was used for acid extraction in the earlier experiments, but later cold 10 % trichloroacetic acid was used as recommended by Schneider (1945) for fractionation of P in animal tissue.) Total and inorganic P were determined on the acid extract, total P on the ethanol-ether extract and on the fibre residue.

The amount of inorganic P in nettle fibre is remarkably high but even higher values of up to 1·2 % of the dry matter have been found. The highest value for tobacco fibre was less than 0·4 % and these plants had been given a large amount of phosphate fertilizer. The P content of the fibre of young leaves is higher than that of old even in 'alkaline drift' plants, but the amount of inorganic P is less. Table 4 compares the P distribution in the fibre of old and young nettle leaves picked in June.

Thoday & Evans (1932) stated that the phosphate in *Helianthus tuberosus* was localized in the multi-

Table 3. *Comparison of fractionation of fibre* P *in tobacco and nettle*

	Tobacco			Nettle		
Fraction	P as % total dry wt.	Total P (%)	Acid-extractable P (%)	P as % total dry wt.	Total P (%)	Acid-extractable P (%)
Total	0·240	100	—	0·720	100	—
Extracted by 0·2 N-HCl	0·103	43	100	0·634	88	100
Inorganic P in acid extract	0·072	17	70	0·570	79	90
Ethanol-ether (7 : 3) extractable	0·024	10	—	0·043	6	—
Residue	0·113	47	—	0·043	6	—

Table 4. *Comparison of* P *fractionation in young and old bryony leaves*

	Young leaves			Old leaves		
Fraction	P as % total dry wt.	Total P (%)	Acid-extractable P (%)	P as % total dry wt.	Total P (%)	Acid-extractable P (%)
Total	0·650	100	—	0·420	100	—
Extracted by 10 % trichloroacetic acid	0·514	79	100	0·294	70	100
Inorganic P	0·108	16·6	21	0·265	63	90
Ethanol-ether (7 : 3) extractable	0·059	9	—	0·063	15	—
Residue	0·078	12	—	0·063	15	—

cellular epidermal hairs and in *H. annuus* in the basal cells of the hairs and the cortical cells below. The method they used involved treatment of sections with a solution of ammonium molybdate in HNO_3 which produced a yellow crystalline precipitate with the phosphate in the tissues; this was then reduced to a bluish-black oxide of molybdenum with phenylhydrazine hydrochloride. A microscopical examination of minced fibre of 'alkaline drift' plants showed that there were large numbers of undamaged epidermal hairs. When marrow leaf fibre was treated by the above method it gave a large quantity of yellow crystals which were concentrated to some extent in and around the hairs, but owing to the acidity of the reagent, which must be maintained, much phosphate was extracted from the fibre into solution. It seems probable that much of the calcium and phosphate are localized in the same cells and that in addition to calcium carbonate some of the calcium is present as a calcium phosphate.

Conditions under which a pH *rise takes place with 'non-drifter' fibre*

A pH rise could be obtained with 'non-drifter' fibre by adding $CaCO_3$ and phosphate and, when the necessary conditions had been established, the investigation was continued to find which constituent of fibre, in addition to $CaCO_3$ and phosphate, could be responsible. When tobacco leaf fibre was milled with $CaCO_3$ the pH did not rise above that of the $CaCO_3$ itself (i.e. pH 8). When, however, it was neutralized by soaking in 0·2M-Na_2HPO_4 adjusted to pH 7·9, washed with water and then milled with $CaCO_3$, a pH of 9·3 was obtained. Unless the fibre was thoroughly washed after 'phosphating', the pH rise did not occur, due undoubtedly to the buffering action of the phosphate. 'Phosphated' washed fibre had a P content about twice the original value. Fibre which had been soaked in phosphate solution and milled without $CaCO_3$ showed a rise but the pH was not as high as with the $CaCO_3$. The actual pH reached on milling, with or without $CaCO_3$, was found to depend on the pH of the phosphate solution in which the fibre had previously been soaked. The results of an experiment using nightshade (*Solanum dulcamara*) and tomato fibre are shown in Fig. 1.

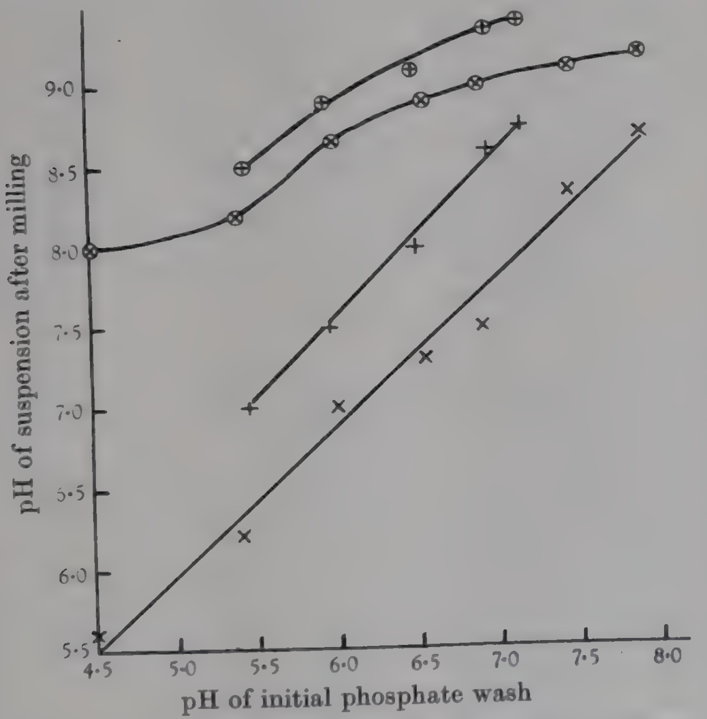

Fig. 1. The effect of milling fibre 'phosphated' at different pH values. ×—× nightshade fibre; ⊗—⊗ nightshade fibre + $CaCO_3$; +—+ tomato fibre; ⊕—⊕ tomato fibre + $CaCO_3$.

A similar effect could be obtained by replacing sodium phosphate as a soaking fluid by sodium citrate or potassium oxalate but not by sodium acetate, sodium sulphate or sodium chloride. The pH rise was also obtained with acid-extracted nettle fibre under the same treatment. Table 5 gives the results of an experiment using minced nettle fibre.

Table 5. *Reconstitution of* pH *rise in acid-extracted nettle fibre*

(15 g. minced nettle fibre soaked in 100 ml. N-HCl overnight, squeezed out and washed twice with 100 ml. water. pH of fibre suspension 2·7.)

Treatment	pH
Fibre (3 g.) milled	5·4
Fibre (3 g.) milled with 1 g. $CaCO_3$	7·4
Fibre (6 g. in 40 ml. 0·2M-Na_2HPO_4) squeezed out and washed	7·9
Fibre (3 g.) milled	8·4
Fibre (3 g.) milled with 1 g. $CaCO_3$	9·7

The constituent of the leaf responsible is not protein since incubation with a commercial trypsin preparation removes a large proportion of the nitrogenous constituents from tobacco fibre but does not prevent the pH rise. Cellulose also does not appear to be involved since experiments with filter paper (treated with alkali to render it suitable for milling, and then well washed) do not give the effect. When, however, pectic acid was removed from fibre it was not possible to obtain a pH rise.

Tobacco fibre (10 g., dry wt. 2·6 g.), suspended in water, was incubated at 40° and pH 5 for 48 hr. with 1 mg. of a preparation of polygalacturonase (purified from a commercial pectinase of fungal origin) to convert pectic to galacturonic acid (Jansen & MacDonnell, 1945). The fibre lost 450 mg. of carbohydrate and, when soaked in neutral phosphate solution, washed and milled with $CaCO_3$, did not show a rise above pH 8. The same effect was brought about by incubation with the crop juice of *Helix aspersa* and was due presumably to its polygalacturonase action. As the enzymic removal of pectic acid from fibre thus abolished the effect, experiments were done with pectic

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acid. This was prepared by de-esterification of citrus pectin (British Drug Houses Ltd., 100 grade) enzymically with tobacco pectase and also with 0·1 N-NaOH. The pectic acid (0·5 g.) was soaked in 25 ml. 0·2 M-Na_2HPO_4 solution for 2 hr. during which a small amount dissolved. The undissolved portion was centrifuged down and then washed three times with 50 ml. water. $CaCO_3$ (0·5 g.) was then added which caused a pH rise to 9, and when the paste of pectic acid and $CaCO_3$ was milled the pH obtained was 9·8. A similar experiment, in which 0·5 g. pectic acid and 0·5 g. $CaCO_3$ were soaked in phosphate solution and the solid material then washed, showed a pH rise to 9·2 by the fourth wash and to 9·9 when milled.

Pectic acid thus behaves in the same way as whole fibre of 'non-drifter' leaves when soaked in phosphate solution, washed and milled with $CaCO_3$, and the phenomenon is similar to that originally observed with the fibre of leaves rich in calcium carbonate and in phosphate.

DISCUSSION

Wilkins (1917) reported very high values for the calcium content of various members of the Cucurbitaceae, but made no suggestion as to its mode of combination. Smith (1944) stated that 70 % of the calcium of squash leaves was immobilized in cell walls or intracellular substances and was readily extracted with dilute acid. It was also observed that the cell wall residue after various extraction treatments when suspended in water had a pH of 9·0.

Although the presence of calcified areas in plant tissue has been known for a considerable time, it has not been observed that a large proportion of the total calcium and as much as 10 % of the dry weight of the leaves of some species is calcium carbonate. The amount of insoluble inorganic phosphate in 'alkaline drift' plants, presumably as calcium phosphate, is also noteworthy.

Two species which show the pH rise, nettle and sunflower, were those investigated by Spoehr and his coworkers from 1923 to 1940 (see Spoehr, Smith, Strain & Milner, 1940; and Smith, 1940) on account of their ability to absorb carbon dioxide when killed by drying or freezing. Smith (1940) concluded that the absorptive agents were calcium and magnesium carbonates, and possibly phosphates.

SUMMARY

1. A pH rise to *c.* 9·5 on washing the fibre of macerated leaves was observed in all species of the Cucurbitaceae examined and some members of the Urticaceae, Boraginaceae and Compositae.

2. The rise is not prevented by heating and is speeded by fine grinding.

3. 'Alkaline drift' plants have a high calcium content, a large proportion of which is insoluble and occurs as calcium carbonate. The calcium content of the fibre may be as high as 11 % of the dry matter and the inorganic phosphorus content is also high, up to 1 % of the dry matter. The calcium and phosphorus appear to be localized in and near the epidermal hairs.

4. Young leaves which have much lower contents of calcium and inorganic phosphorus do not show much pH rise.

5. A pH rise to > 9 can be produced with 'non-drifter' and acid-extracted 'drifter' fibre and with pectic acid by soaking in neutral phosphate solution, washing and milling with calcium carbonate. After incubation of 'non-drifter' fibre with polygalacturonase the pH rise cannot be obtained.

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Effects of Organic Arsenicals on Enzyme Systems

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Investigations have been carried out by us in the Biochemical Laboratory of the Cardiff City Mental Hospital during 1938, 1939 and early 1940 on the effects of organic trivalent and pentavalent arsenic compounds on proteins and enzymes. The work was primarily undertaken to elucidate the biochemical basis of the trypanocidal and anti-spirochaetal action of a variety of organic arsenic compounds used in the treatment of syphilis. Investigations by Hawking, Hennelly & Quastel (1937) had already shown that administration of the pentavalent arsenical tryparsamide into the body led to the appearance of trivalent arsenic compounds, of high trypanocidal activity, in the spinal fluid. On the other hand, administration of organic trivalent arsenic compounds did not lead to the excretion of active trivalent arsenic compounds in the spinal fluid. Work was therefore undertaken to discover the manner of combination of organic trivalent and pentavalent arsenic compounds with tissue proteins and enzyme systems. It was inevitable that our work led us into problems connected with the mechanism of action of certain war gases (e.g. lewisite) and with the question of reversibility of enzyme systems inactivated by arsenic compounds. Whilst we, ourselves, did not work with actual war gases, our results bore on the problems involved and for this reason publication of our results was not allowed during the war. A report, however, was submitted to the Medical Research Council in November 1940, and an extract containing the main conclusions was published in *Nature* (Gordon & Quastel, 1947) by permission of the Secretary of the Medical Research Council.

The present paper contains the results of investigations made by us on the effects of organic arsenic compounds on enzyme systems during 1938, 1939 and early 1940; they are the results on which the conclusions, already published by Gordon & Quastel (1947), are based.

Much attention has been devoted by workers in chemotherapy to the elucidation of the mechanism of the trypanocidal and anti-spirochaetal activity of arsenic compounds. It is a well established fact, first noted by Ehrlich, that while pentavalent arsenic components are relatively non-toxic, *in vitro*, to protozoa, trivalent arsenic compounds such as substituted phenylarsenoxides and arsenobenzenes are extremely toxic. Moreover, there is good reason to believe that, in the case of the arsenobenzenes, the toxic action is not exerted to any great extent by these compounds as such, but is probably due to the presence, in their aqueous solutions, of highly toxic arsenoxides, rapidly formed by atmospheric oxidation of the —As=As— group. Eagle (1939*a*) has shown that neoarsphenamine is 25–60 times more toxic to trypanosomes under aerobic conditions than it is when strict anaerobic conditions are observed, the change taking place within 2–5 min. of dissolving the compound; there is no difference of this kind shown in the case of mapharside (*m*-amino-*p*-hydroxyphenylarsenoxide). It would appear, therefore, that the arsenoxides are the most highly toxic of the aromatic arsenic compounds, and much experimental evidence has accumulated which has led to the probability that this toxicity is due to the ability of the arsenoxides to react with thiol compounds.

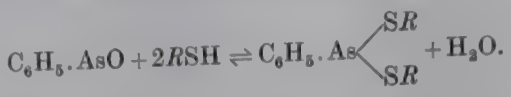
This conclusion was anticipated by Ehrlich (1909) when he suggested that thiol groups could act as chemo-receptors for trivalent arsenic compounds. Later, Voegtlin, Dyer & Leonard (1923) postulated that the toxic action of an arsenoxide to trypanosomes is due to a condensation with thiol groups present in protoplasm, leading to a dysfunction of the system regulating cell respiration. The fact that arsenic compounds are capable of combining with organic compounds containing thiol groups (such as thiolacetic acid or thioglycollic acid) was demonstrated by Barber (1929), who showed that a condensation takes place between phenylarsenoxides and thiol-substituted compounds, giving a derivative (arylthioarsenite or thioglycollate) according to the equation

$$C_6H_5.AsO+2RSH=C_6H_5.As\left\langle\begin{matrix}SR\\SR\end{matrix}\right.+H_2O.$$

Barber also showed that thiol compounds in sufficient concentration will reduce a phenylarsonic acid to a phenylarsenoxide,

$$C_6H_5.AsO_3H_2+2RSH\rightarrow C_6H_5.AsO+RSSR+2H_2O,$$

which then combines with excess of thiol compound to give an arylthioarsenite as above. Gough & King (1930) subsequently deduced, from a scrutiny of the properties and reactions of arylthioarsenites, that these derivatives are not stable, but are slowly hydrolyzed at ordinary temperatures even in neutral solution, with liberation of small amounts of toxic arsenoxides. This supposition supported their view, that when pentavalent phenylarsonic acid derivatives are administered therapeutically in the treatment of protozoal infections, reduction takes place in the tissues to give arsenoxides, which then condense with thiol compounds of the tissues, and are 'stored' in this manner. The highly trypanocidal arsenoxides are finally set free, by hydrolysis, over an extended period of time, and are responsible for the prolonged therapeutic effect of the drug. It would appear, therefore, that, in neutral solution, an equilibrium can exist between an arsenoxide and a thiol compound, as follows:



$$C_6H_5.AsO + 2RSH \rightleftharpoons C_6H_5.As\begin{matrix}SR\\SR\end{matrix} + H_2O.$$

If it is true that the toxicity of arsenic compounds to micro-organisms is due to a combination between arsenoxide and thiol groups, causing a breakdown in some essential metabolic process, then it would be reasonable to suppose that, in presence of excess of added thiol compound, the toxicity of the arsenic compound would be greatly reduced, since the arsenic would then be present in the system mainly in the form of arylthioarsenite, which should be non-toxic since it is incapable of further reaction with thiol groups. Such an effect was reported by Eagle (1939*b*) who discovered that, in presence of sufficient excess of thiol compounds such as cysteine, glutathione, etc., the anti-spirochaetal activity of trivalent arsenic compounds is almost completely removed; no effect of this sort was noticed when methionine was used. The large excess of thiol compound necessary to produce the effect was in harmony with the views of Gough & King (1930) upon the hydrolysis of the arylthioarsenites.

In order to obtain more information regarding the capacity of trivalent arsenic compounds to interfere with metabolic processes by virtue of their affinity for thiol groups, we considered it advisable to undertake an investigation of the manner in which arsenic compounds may influence the activities of a variety of enzyme systems. Evidence has been obtained by a number of workers that certain enzymes require, for their activity, the integrity of thiol groups in their structure (see review of earlier work by Hellerman, 1937). Toxicity of trivalent arsenoxides to enzymes may throw light on, or confirm, the thiol nature of such enzymes. The experimental results which are now reported provide evidence confirming the conclusions of earlier workers on the thiol structure of certain enzymes, and indicating for the first time the thiol structure of choline dehydrogenase. At the same time evidence will be given clearly demonstrating the reactivation by thiol compounds of enzyme systems inhibited or poisoned by arsenic compounds.

EXPERIMENTAL METHODS

Urease

Preparation and measurement of activity. A commercial (Dunning) urease tablet (25 mg.) was ground in 5 ml. saline. CO_2 gas was passed through the suspension for a few minutes at room temperature and the mixture was centrifuged. The clear supernatant fluid had high urease activity and 0·2–0·4 ml. of this fluid was used for the enzyme studies.

A manometric technique (Barcroft or Warburg) was exclusively used. The urease preparation was introduced into the main compartment of the vessel of a Warburg or Barcroft manometric apparatus in presence of 0·13 M-sodium acetate buffer, pH 5·0, at 37°. Urea (0·2 ml. of 10% solution) was placed in the side tube of the manometer vessel, and after thermal equilibrium the urea was tipped into the main vessel and readings of CO_2 output were noted at frequent intervals as the urea decomposed. The rate of CO_2 output was a measure of the activity of the urease preparation.

Additions of organic arsenic compounds and other inhibitors. These substances were added to the urease preparation in the main manometer vessel prior to the addition of urea. Thiol compounds, or other substances under investigation, were also added to the urease in the main compartment of the Warburg or Barcroft manometer vessel before addition of the urea. Any divergence from this procedure is noted in the text.

Addition of animal tissues. Experiments were carried out to determine whether the presence of animal tissues would inactivate organic arsenic compounds. Usually, known quantities of the arsenical were incubated with about 1 g. animal tissue (e.g. liver slices, or blood) at 37° for 2 hr. in presence of 0·037 M-$NaHCO_3$ solution in an atmosphere of 7% CO_2 + 93% O_2. At the end of this period 1 ml. of the supernatant was transferred to the acetate-urease mixture present in a Warburg manometric vessel. After thermal equilibrium, urea was tipped in from a side tube and the course of CO_2 evolution was followed. Control experiments in the absence of tissues were always set up.

Hydrolytic enzymes

Cholinesterase. Whole fresh rat brain (1·5 g.) was ground with sand in the presence of saline (8 ml.). The fine suspension was filtered through washed muslin and 0·5 ml. of the filtrate was used for the majority of estimations.

Activity measurements were carried out in either the Barcroft or Warburg manometric apparatus. The enzyme preparation was placed in the main compartment of a manometric vessel in presence of 0·027 M-$NaHCO_3$ solution in an atmosphere of 5% CO_2 + 95% N_2. Acetylcholine chloride (0·2 ml. of a 2% solution in $NaHCO_3$ solution) was placed in a side tube of the apparatus and this was tipped into the main vessel after thermal equilibrium had been set up. The rate of CO_2 evolution was a measure of the activity of the cholinesterase preparation.

Liver esterase. Rat-liver tissue was used as a source of liver esterase either in the form of thin, intact slices of fresh liver or as a suspension made by mincing 1 g. fresh liver in 15 ml. saline, 0·2–0·5 ml. of the suspension being usually employed. Activity was measured as with cholinesterase, with 0·2 ml. tributyrin as substrate. Control experiments without tissue were always set up.

Pancreatic lipase or esterase. Commercial pancreatin was used as a source of the enzyme and 0·5 ml. of a 1 in 100 suspension of pancreatin in saline was usually employed, with 0·2 ml. tributyrin as substrate. Activities were estimated as with cholinesterase.

Invertase. Commercial invertase was used as a source of the enzyme. The preparation (0·1 g.) was ground in 10 ml. water and the suspension was centrifuged. Of the clear centrifugate 1 ml. was usually employed. The general procedure for estimating the effects of organic arsenicals was as follows: In a series of tubes were placed 5 ml. 40% sucrose solution, 3 ml. 0·2 M-sodium acetate buffer solution, pH 5·0, and 1 ml. of either water or an aqueous solution of the organic arsenical. The mixtures were placed in a water bath at 40°, and after 30 min. 1 ml. of the invertase preparation was added to each tube. At known intervals, pairs of tubes were removed from the water bath, and the contents were treated with 2 ml. of 6% trichloroacetic acid and filtered. To 8 ml. of the filtrate 2 ml. N-NaOH were added, and the mixture was transferred to a 1 dm. polarimeter tube and the optical rotation read. The course of inversion of sucrose was followed in this way and estimates were made of the effects of the organic arsenicals on the invertase activity.

Oxidation systems

The activities of these systems were measured either (*a*) under aerobic conditions by estimating the rates of O_2 uptake when tissues, or their extracts, respired in air or O_2 in a Locke 0·03 M-sodium phosphate pH 7·4 medium either in the presence or absence of organic arsenicals, or (*b*) under anaerobic conditions by means of the ferricyanide technique of Quastel & Wheatley (1938) whereby the rate of evolution of CO_2 produced from bicarbonate on reduction of ferricyanide, in an atmosphere of 5% CO_2 + 95% N_2, was followed.

Succinic dehydrogenase. Rat liver, or rat kidney, slices were used as sources of this enzyme when intact tissues were investigated. A purified preparation of succinic dehydrogenase made from pig heart by the method of Keilin & Hartree (1940) was also used. In Exp. 6, Table 4, 0·5 ml. of a saline suspension of minced sheep liver (1 g./5 ml. saline) was employed.

Choline dehydrogenase. This enzyme was used either in the form of 1 ml. of a saline suspension of washed, minced rat liver (1 g. liver/5 ml. saline) or as intact liver slices (25 mg. dry weight).

Cytochrome oxidase. A saline suspension (1 ml.) of minced washed rat liver was generally used.

Lactic dehydrogenase. Washed human red blood cells lysed with water (1 part of cells/3 parts water) were used as a source of this enzyme. 1 ml. of the lysed preparation, in presence of cozymase (5 mg.) and neutral NaCN (0·08 M), was usually employed.

Oxidations of glucose, lactate, or pyruvate. These oxidation systems were studied with the aid of saline suspensions of minced rat brain (1 g. whole brain/5 ml. saline), 1 ml. of the saline suspension being used.

Catalase. Minced rat liver (1 g.) was shaken with 100 ml. saline and the mixture centrifuged. The clear centrifugate served as a source of catalase, 0·5 ml. of the fluid being used.

RESULTS

Urease

Effects of a variety of organic arsenic compounds in the presence or absence of thiol compounds are shown in Tables 1 and 2 and Figs. 1 and 2.

The results show:

(1) Arsenoxides, in the form of phenylarsenoxide, mapharside (*m*-amino-*p*-hydroxyphenylarsenoxide), *p*-acetamidophenylarsenoxide, *m*-acetamido-*p*-carboxyphenylarsenoxide, and the products of condensation of thioglycollic acid with phenylarsenoxide and the reduced forms of atoxyl and tryparsamide have highly inhibitory effects on urease activity. The effects exceed those due to sodium arsenite at equivalent concentrations.

(2) Stabilarsan (the diglucoside of arsphenamine) has weakly toxic effects on urease, less than those of sodium arsenite at equivalent concentrations.

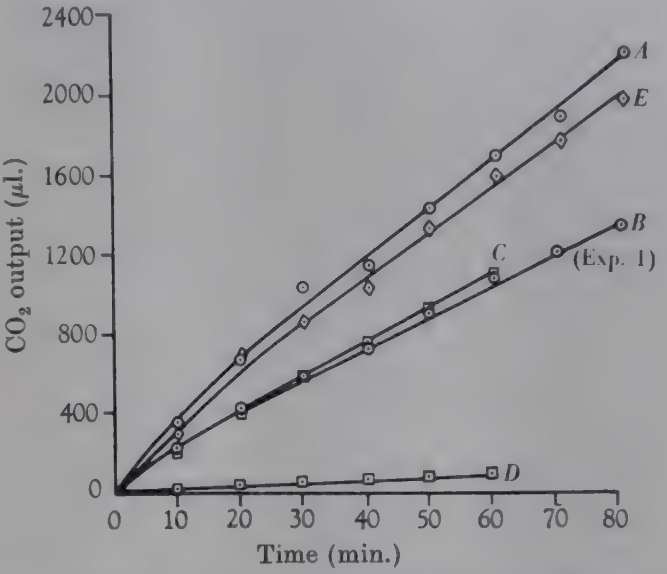


Fig. 1. Effects of arsenic compounds on urease activity at 37° and an acidity of pH 5. Exp. 1, Table 2: *A*, urease control; *B*, urease + 33 µg./ml. mapharside. Exp. 3, Table 2: *C*, urease control; *D*, urease + 67 µg./ml. phenylarsenoxide thioglycollate. Exp. 7, Table 2: *A*, urease control; *E*, urease + 670 µg./ml. tryparsamide.

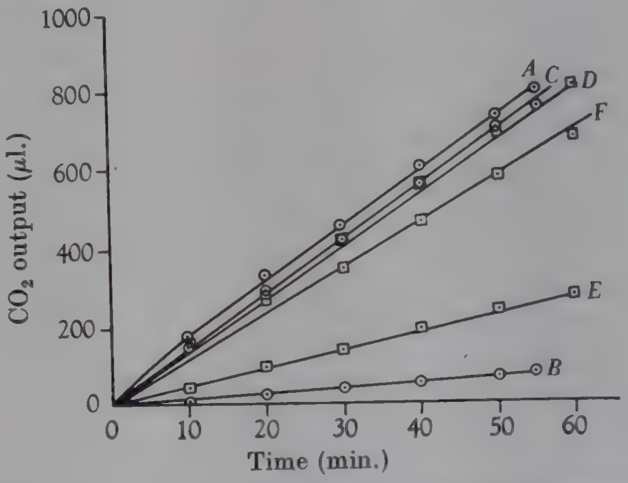


Fig. 2. Effects of arsenic compounds with and without added cysteine on urease activity at 37° and an acidity of pH 5. Exp. 14, Table 2: *A*, urease control; *B*, urease + 67 µg./ml. phenylarsenoxide thioglycollate; *C*, urease + 67 µg./ml. phenylarsenoxide thioglycollate + 2 mg./ml. cysteine. Exp. 15, Table 2: *D*, urease control; *E*, urease + 67 µg./ml. reduced tryparsamide thioglycollate; *F*, urease + 67 µg./ml. reduced tryparsamide thioglycollate + 2 mg./ml. cysteine.

(3) The presence of excess of a thiol compound, such as cysteine or glutathione, eliminates or greatly reduces the toxic effect of arsenoxides or of condensation products of arsenoxides and thioglycollic acid.

(4) Excess of NaCN does not affect the toxicity of an arsenoxide to urease.

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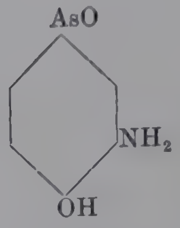
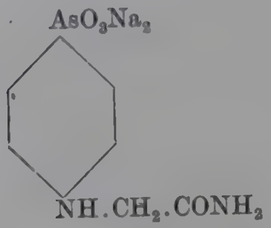
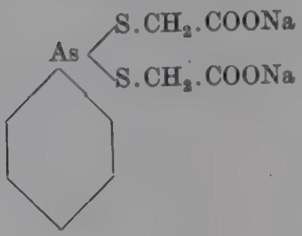
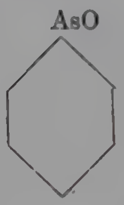
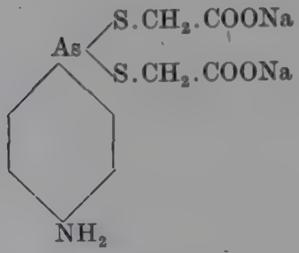
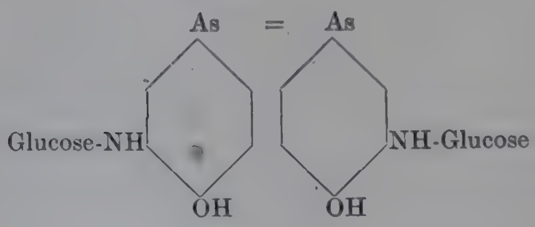
Table 1. *Effects of arsenic compounds on urease at 37° and an acidity of* pH 5·0

Exp.	Arsenic compound added	Constitution	Conc. (M)	Enzyme activity (CO_2 output, µl./hr.)	Inhibition of enzyme activity (%)
1	Nil		—	1665	—
	Mapharside		$1{\cdot}6 \times 10^{-4}$	1020	39
	Tryparsamide		$2{\cdot}1 \times 10^{-3}$	1491	10
2	Nil		—	1120	—
	Phenylarsenoxide thioglycollate		$1{\cdot}8 \times 10^{-4}$	85	92
3	Nil		—	1264	—
	Phenylarsenoxide		$4{\cdot}0 \times 10^{-4}$	408	68
4	Nil		—	1308	—
	Sodium arsenite	Na_3AsO_3	$0{\cdot}86 \times 10^{-3}$	876	33
	Sodium arsenite		$1{\cdot}73 \times 10^{-3}$	609	54
5	Nil		—	1245	—
	Reduced atoxyl thioglycollate		$2{\cdot}55 \times 10^{-4}$	672	47
6	Nil		—	1152	—
	Stabilarsan		$0{\cdot}97 \times 10^{-3}$	1008	13
	Stabilarsan		$2{\cdot}4 \times 10^{-3}$	768	34

(5) Toxicity of an arsenoxide to urease does not increase with time, indicating the probable existence of an equilibrium between the arsenoxide and the enzyme.

(6) The presence of tissues such as liver slices, or of whole or lysed blood, diminishes toxicity of arsenoxides to urease. This is due to the fact that the effective concentration of arsenoxide is diminished by combination of the arsenical with the tissue proteins.

(7) The pentavalent arsenic compound, tryparsamide, shows little toxicity to urease.

These conclusions confirm the observations made by previous authors as to the inhibitory effects of

Table 2. *Effects of organic arsenic compounds on urease*

Exp.	Arsenic compound	Conc. of arsenic compound (μg./ml.)	Duration of exp. (min.)	Total CO_2 output (μl.)	Inhibition of enzyme activity (%)
1	Nil	—	80	2220	—
	Mapharside	33	80	1360	39
2	Nil	—	50	633	—
	Mapharside	67	50	160	74·7
	Nil + NaCN (2 mg./ml.)	—	50	627	<1
	Mapharside + NaCN (2 mg./ml.)	67	50	163	74·2
3	Nil	—	60	1120	—
	Phenylarsenoxide thioglycollate	67	60	85	92
4	Nil	—	30	390	—
	Phenylarsenoxide thioglycollate	67	30	20	95
5	Nil	—	40	830	—
	Reduced atoxyl thioglycollate	100	40	449	46
6	Nil	—	40	830	—
	Reduced atoxyl thioglycollate	200	40	363	56
7	Nil	—	80	2220	—
	Tryparsamide	670	80	1990	10
8	Nil	—	30	281	—
	Tryparsamide	1300	30	279	<1
9	Nil	—	15	218	—
	p-Acetamidophenylarsenoxide	33	15	195	10·6
10	Nil	—	15	215	—
	m-Acetamido-*p*-carboxyphenylarsenoxide	33	15	7	96·7
	Effects of addition of cysteine and glutathione				
11	Nil	—	55	837	—
	Nil + cysteine (2 mg./ml.)	—	55	767	8·4
	Mapharside	67	55	299	64·4
	Mapharside + cysteine (2 mg./ml.)	67	55	761	9·1
12	Nil	—	70	682	—
	Nil + cysteine (2 mg./ml.)	—	70	625	8·4
	Mapharside	67	70	216	68·3
	Mapharside + cysteine (2 mg./ml.)	67	70	591	13·3
13	Nil	—	60	852	—
	Nil + cysteine (2 mg./ml.)	—	60	867	—
	Phenylarsenoxide thioglycollate	67	60	49	94·7
	Phenylarsenoxide thioglycollate + cysteine (2 mg./ml.)	67	60	612	28·2
14	Nil	—	55	809	—
	Phenylarsenoxide thioglycollate	67	55	77	90·5
	Phenylarsenoxide thioglycollate + cysteine (2 mg./ml.)	67	55	761	5·9
15	Nil	—	60	822	—
	Reduced tryparsamide thioglycollate	67	60	278	66·2
	Reduced tryparsamide thioglycollate + cysteine (2 mg./ml.)	67	60	687	16·4
16	Nil	—	45	780	—
	Nil + glutathione (2 mg./ml.)	—	45	741	5
	Mapharside	67	45	185	76·3
	Mapharside + glutathione (2 mg./ml.)	67	45	752	3·6
	Effects of addition of animal (rat) tissues				
17	Nil	—	30	694	—
	Nil + liver slices (330 mg./ml.)	—	30	642	7
	Mapharside	22	30	359	48
	Mapharside + liver slices (330 mg./ml.)	22	30	654	6
18	Nil	—	25	916	—
	Nil + whole blood (0·17 ml./ml.)	—	25	907	1
	Mapharside	33	25	464	49
	Mapharside + whole blood (0·17 ml./ml.)	33	25	821	13
19	Nil	—	30	1070	—
	Nil + lysed blood (0·17 ml./ml.)	—	30	1310	—
	Mapharside	33	30	452	58
	Mapharside + lysed blood (0·17 ml./ml.)	33	30	1390	—

Table 3. *Effects of organic arsenic compounds with and without the addition of cysteine on hydrolytic enzymes*

Exp.	Source of enzyme	Arsenic compound added	Conc. of arsenic compound (μg./ml.)	Duration of exp. (min.)	CO_2 output (μl.)	Inhibition of enzyme activity (%)
		I. Cholinesterase (substrate: acetylcholine 1·3 mg./ml.)				
1	Rat brain suspension (1 ml.)	Nil	—	60	583	—
		Mapharside	500	60	369	36·7
		3-Amino-4-hydroxy-phenylarsonic acid	1300	60	502	14
2	Rat brain suspension (0·5 ml.)	Nil	—	60	373	—
		Mapharside	500	60	209	46
		3-Amino-4-hydroxy-phenylarsonic acid	600	60	347	8
3	Rat brain suspension (0·5 ml.)	Nil	—	100	459	—
		Mapharside	330	100	273	41
		Mapharside + cysteine (10 mg./ml.)	330	100	359	22
4	Rat brain suspension (0·5 ml.)	Nil	—	60	292	—
		Mapharside	330	60	170	41
		Mapharside + cysteine (10 mg./ml.)	330	60	226	22
5	Rat brain suspension (0·5 ml.)	Nil	—	70	501	—
		Nil + cysteine (10 mg./ml.)	—	70	503	—
		II. Liver esterase (substrate: tributyrin 700 μg./ml.)				
6	Rat liver slices (80 mg.)	Nil	—	25	1120	—
		Mapharside	270	25	613	45
7	Rat liver slices (50 mg.)	Nil	—	35	761	—
		Mapharside	270	35	295	61
		Tryparsamide	700	35	543	27
8	Minced liver suspension	Nil	—	20	1250	—
		Tryparsamide	700	20	555	68
9	Minced liver suspension	Nil	—	15	946	—
		Atoxyl	700	15	174	81
10	Minced liver suspension	Nil	—	40	1250	—
		Nil + cysteine (700 μg./ml.)	—	40	934	25·2
		Mapharside	330	40	306	71·2
		Mapharside + cysteine (700 μg./ml.)	330	40	680	45·6
11	Minced liver suspension	Nil	—	35	1070	—
		Nil + cysteine (800 μg./ml.)	—	35	760	29
		Mapharside	100	35	424	60·4
		Mapharside + cysteine (800 μg./ml.)	100	35	635	40·7
		III. Pancreatic lipase (substrate: tributyrin 700 μg./ml.)				
12	Pancreatin suspension	Nil	—	15	633	—
		Mapharside	330	15	628	0·8
13	Pancreatin suspension	Nil	—	20	963	—
		Tryparsamide	700	20	872	9
14	Pancreatin suspension	Nil	—	20	837	—
		Atoxyl	700	20	805	4
		IV. Invertase (substrate: 20% sucrose)			Change of optical rotation (°)	
15	Commercial yeast invertase solution	Nil	—	60	11·23	—
		Mapharside	200	60	10·78	4
16	Commercial yeast invertase solution	Nil	—	90	12·26	—
		Mapharside	200	90	12·11	1
17	Commercial yeast invertase solution	Nil	—	45	2·92	—
		Mapharside	200	45	2·85	2
18	Commercial yeast invertase solution	Nil	—	70	3·82	—
		Mapharside	200	70	3·81	0

substances (e.g. heavy metals, quinones, etc.) which react with thiol groups (Quastel, 1933; Perlzweig, 1932; Hellerman, Perkins & Clarke, 1933; Sumner & Poland, 1933; Smythe, 1936; Weiss, 1937) and support the conclusion that urease depends for its activity on the presence of thiol groups in its structure.

Pentavalent arsenic compounds become appreciably toxic only when reduction to the trivalent arsenoxide occurs, and this toxicity may be eliminated either by combination with thiol compounds, which must be in excess to prevent too great a dissociation of the easily reversible arsenoxide-thiol complex, or by combination with tissue proteins.

Hydrolytic enzymes

Cholinesterase. Typical results showing the effects of arsenicals on cholinesterase are given in Table 3 and Fig. 3. These results demonstrate:

(1) Cholinesterase is inhibited by the trivalent mapharside but not, or only feebly, by the pentavalent 3-amino-4-hydroxyphenylarsonic acid.

(2) The effect of the arsenoxide on the enzyme does not increase with time, showing the existence of an equilibrium between arsenical and enzyme.

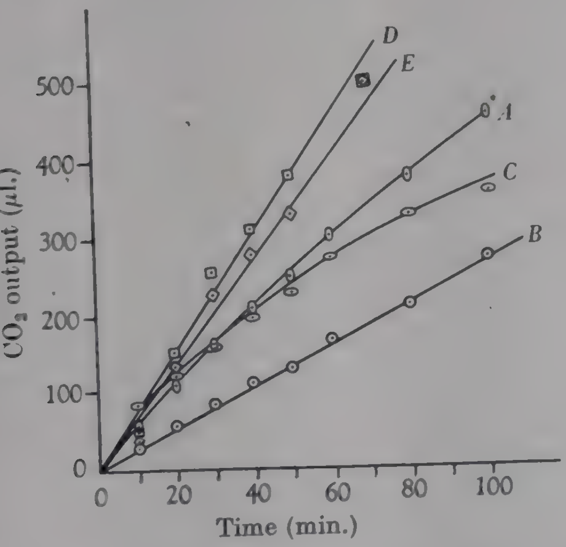


Fig. 3. Effects of arsenic compounds with and without added cysteine on cholinesterase at 37°. Exp. 3, Table 3: *A*, cholinesterase control; *B*, cholinesterase + 330 µg./ml. mapharside; *C*, cholinesterase + 330 µg./ml. mapharside + 10 mg./ml. cysteine. Exp. 5, Table 3: *D*, cholinesterase control; *E*, cholinesterase + 10 mg./ml. cysteine.

(3) The inhibitory action of the arsenoxide is greatly diminished by the presence of excess of cysteine.

The facts support the view that cholinesterase is a thiol enzyme (Nachmansohn & Lederer, 1939).

Liver esterase. Results obtained with this enzyme are shown in Table 3. Liver esterase is exceptional, among the enzymes which have been investigated, in being inhibited by pentavalent as well as by trivalent arsenic compounds. Thus tryparsamide and atoxyl exert toxic effects which are comparable with those due to mapharside. The toxic effect of atoxyl has already been noted by Jowett & Quastel (1935). The inhibitory effects of mapharside are not greatly diminished, or nullified, by the addition of excess of cysteine. A diminution in toxicity does take place, but it is difficult to assess, since cysteine alone exerts inhibitory effects on the activity of the liver esterase preparations we have used.

The facts we have obtained indicate the sensitivity of the enzyme preparation to the presence of arsenic whether this is in the trivalent or pentavalent condition. It would be wise to suspend judgement as to the thiol nature of the enzyme until a purified preparation of liver esterase has been investigated.

Pancreatic esterase. This enzyme preparation, in contrast to that of liver esterase, showed little or no sensitivity to trivalent or pentavalent organic arsenicals, under the conditions of our experiments.

Invertase. This enzyme is not attacked by mapharside at the concentrations tried (see Table 3), and is therefore unlikely to be a thiol enzyme.

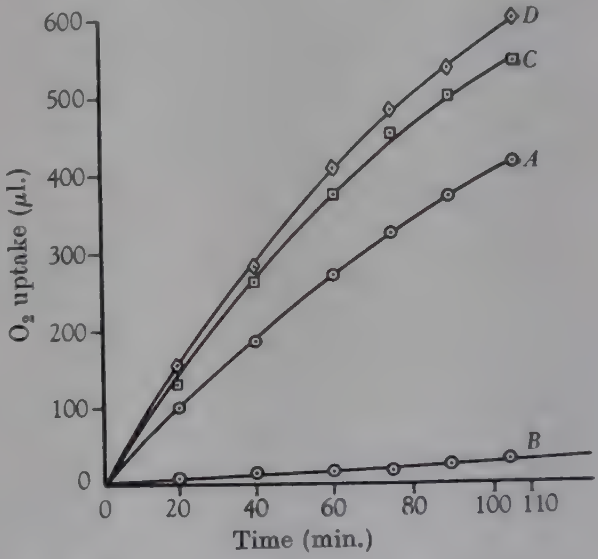


Fig. 4. Effects of mapharside, with and without added glutathione, on succinic dehydrogenase activity. Exp. 4, Table 4: *A*, succinic dehydrogenase control; *B*, succinic dehydrogenase + 2·7 µg./ml. mapharside; *C*, succinic dehydrogenase + 700 µg./ml. glutathione; *D*, succinic dehydrogenase + 2·7 µg./ml. mapharside + 700 µg./ml. glutathione.

Oxidation systems

Succinic dehydrogenase. Results in Table 4 and Fig. 4 show the highly inhibitory effects of mapharside on the activity of succinic dehydrogenase and the lack of toxic action of tryparsamide. The poisoning effect of the trivalent arsenical is nullified by the presence of an excess of glutathione. These

Table 4. *Effects of organic arsenic compounds on oxidizing enzymes in presence and absence of thiol compounds*

Exp.	Source of enzyme	Arsenic compound added	Conc. of arsenic compound (µg./ml.)	Duration of exp. (min.)	O_2 uptake (µl.)	Inhibition of enzyme activity (%)
		I. Succinic dehydrogenase (substrate: sodium succinate M/30)				
1	Rat liver slices (100 mg.)	Nil	—	30	596	—
		Mapharside	70	30	269	58
2	Rat kidney slices (100 mg.)	Nil	—	130	581	—
		Mapharside	133	130	192	67
3	Rat kidney slices (100 mg.)	Nil	—	70	539	—
		Tryparsamide	7000	70	536	0
4	Pig heart extract (0·5 ml.)	Nil	—	105	414	—
		Mapharside	2·7	105	28	93
		Nil + glutathione (700 µg./ml.)	—	105	544	—
		Mapharside + glutathione (700 µg./ml.)	2·7	105	597	0
5	Pig heart extract (0·5 ml.)	Nil	—	60	470	—
		Mapharside	2·7	60	167	64
		Nil + glutathione (700 µg./ml.)	—	60	544	—
		Mapharside + glutathione (700 µg./ml.)	2·7	60	535	0
					Anaerobic CO_2 output (µl.)	
6	Sheep liver extract (0·5 ml.)	Nil	—	30	599	—
		Mapharside	170	30	137	77
7	Rat liver slices (100 mg.)	Nil	—	60	917	—
		Mapharside	133	60	264	71
		II. Choline dehydrogenase (substrate: choline M/30)			O_2 uptake (µl.)	
8	Rat liver suspension (1 ml.)	Nil	—	40	388	—
		Reduced tryparsamide thioglycollate	170	40	212	45
		Reduced tryparsamide thioglycollate	330	40	115	70
9	Rat liver suspension (1 ml.)	Nil	—	60	626	—
		Reduced tryparsamide thioglycollate	230	60	249	60
		Tryparsamide	1300	60	586	6
10	Rat liver suspension (1 ml.)	Nil	—	60	228	—
		Mapharside	200	60	65	71
		Mapharside + cysteine (5 mg./ml.)	200	60	223	0
					Anaerobic CO_2 output (µl.)	
11	Rat liver slices (25 mg. dried tissue)	Nil	—	45	791	—
		Mapharside	130	45	388	50
12	Rat liver suspension (1 ml.)	Nil	—	60	707	—
		Mapharside	130	60	463	34
13	Rat liver slices (25 mg. dried tissue)	Nil	—	60	640	—
		Mapharside	170	60	212	67
		Mapharside + cysteine (5 mg./ml.)	170	60	539	16
		III. Cytochrome oxidase (substrate: *p*-phenylenediamine 3·3 mg./ml.)			O_2 uptake (µl.)	
14	Rat liver suspension (1 ml.)	Nil	—	40	472	—
		Mapharside	133	40	480	0
		Mapharside	330	40	466	1

Table 4 (*cont.*)

Exp.	Source of enzyme	Arsenic compound added	Conc. of arsenic compound (μg./ml.)	Duration of exp. (min.)	Anaerobic CO_2 output (μl.)	Inhibition of enzyme activity (%)
		IV. Lactic dehydrogenase (substrate: sodium lactate 16 mg./ml.)				
15	Lysed human red blood cells (1 blood : 3 water) (1 ml.) + cozymase + NaCN (neutral, M/12)	Nil	—	60	639	—
		Mapharside	330	60	598	6
		V. Glucose oxidation (substrate: glucose 0·01 M)				
18	Rat brain suspension (1 ml.)	Nil	—	150	349	—
		Mapharside	170	150	176	50
19	Rat brain suspension (1 ml.)	Nil	—	180	476	—
		Mapharside	170	180	99	79
		Tryparsamide	1700	180	650	0
20	Rat brain suspension (1 ml.)	Nil	—	120	583	—
		Mapharside	170	120	264	55
		Mapharside + cysteine (3·3 mg./ml.)	170	120	567	3
21	Rat brain suspension (1 ml.)	Nil	—	180	317	—
		3-Acetamido-4-carboxy-phenylarsenoxide	33	180	241	24
		3-Acetamido-4-carboxy-phenylarsenoxide + cysteine (660 μg./ml.)	33	180	319	0
		VI. Pyruvate oxidation (substrate: sodium pyruvate 0·01 M)				
22	Rat brain suspension (1 ml.)	Nil	—	150	360	—
		Mapharside	200	150	95	74
23	Rat brain suspension (1 ml.)	Nil	—	180	634	—
		Nil + glutathione (5 mg./ml.)	—	180	596	6
		Mapharside	200	180	12	98
		Mapharside + glutathione (5 mg./ml.)	200	180	420	33
24	Rat brain slices	Nil	—	110	185	—
		Mapharside	200	110	74	60
		VII. Lactate oxidation (substrate: sodium lactate 0·02 M)				
25	Rat brain suspension (1 ml.)	Nil	—	60	200	—
		Mapharside	133	60	3	98
		Mapharside	67	60	27	86
26	Rat brain suspension (1 ml.)	Nil	—	150	463	—
		Nil + glutathione (330 μg./ml.)	—	150	452	2
		Mapharside	17	150	25	94
		Mapharside + glutathione (330 μg./ml.)	17	150	363	21
		VIII. Catalase (substrate: H_2O_2, 0·03 %)			O_2 output (μl.)	
27	Rat liver extract (0·5 ml.)	Nil	—	40	621	—
		Mapharside	133	40	614	1
		Mapharside	330	40	556	10

facts point to the thiol nature of succinic dehydrogenase, confirming the observations of Hopkins & Morgan (1938), Hopkins, Morgan & Lutwak-Mann (1938) and of Keilin & Hartree (1940). The greater the purity of the enzyme preparation the greater is the toxicity secured by a given concentration of arsenoxide. This is due to the purified preparation containing smaller quantities of tissue proteins which compete with the enzyme for the arsenical.

Choline dehydrogenase. This enzyme (for details of properties and estimation see Mann & Quastel, 1937; Bernheim & Webster, 1937; Mann, Woodward & Quastel, 1938) is highly sensitive to such trivalent organic arsenic compounds as mapharside and reduced tryparsamide thioglycollate. The pentavalent tryparsamide, however, does not inhibit the enzyme. The presence of cysteine, in excess, reverses the inhibitory action of mapharside on choline

Table 5. *Effects of various inhibitors on choline dehydrogenase*

(Substrate: choline 0·033 M)

Exp.	Source of enzyme	Inhibitor	Conc. of inhibitor (M)	Duration of exp. (min.)	O_2 uptake (µl.)	Inhibition of enzyme activity (%)
1	Saline suspension of washed minced rat liver	Nil (choline absent)	—	60	167	—
		Sodium tetrathionate (choline absent)	2×10^{-4}	60	102	—
		Nil	—	60	621	—
		Sodium tetrathionate	2×10^{-4}	60	382	38
2	Saline suspension of washed minced rat liver	Nil (choline absent)	—	60	75	—
		Sodium tetrathionate (choline absent)	6×10^{-4}	60	43	—
		Nil	—	60	549	—
		Sodium tetrathionate	6×10^{-4}	60	80	93
3	Saline suspension of washed minced rat liver	Nil (choline absent)	—	60	89	—
		Alloxan (choline absent)	0·22	60	31	—
		Nil	—	60	508	—
		Alloxan	0·22	60	107	82
4	Saline suspension of washed minced rat liver	Nil	—	60	482	—
		Alloxan	0·022	60	90	82
5	Saline suspension of washed minced rat liver	Nil (choline absent)	—	150	63	—
		Nil	—	150	317	—
		Oxidized glutathione	0·05	150	204	44

dehydrogenase. Results supporting these conclusions are shown in Table 4 and Figs. 8 and 9. These results lead to the view that choline dehydrogenase is a thiol enzyme. This conclusion is supported by the fact that the following compounds also inhibit choline dehydrogenase, viz. sodium tetrathionate, alloxan, and oxidized glutathione. Typical results are shown in Table 5. These substances, as shown by Hopkins & Morgan (1938) and by Keilin & Hartree (1940), have inhibitory effects on succinic dehydrogenase, presumably by oxidation of the thiol groups of the enzyme.

Pyruvic oxidase. The oxidation of pyruvic acid by minced rat brain is very greatly inhibited by mapharside, the inhibition being suppressed by the presence of excess of glutathione (Table 4, and Fig. 6). These facts point to the thiol nature of pyruvic oxidase, already suggested by Peters (1937) and by experiments of Barron (1936) on pyruvate oxidation of gonococci.

Glucose and lactate oxidation. The respiration of minced rat brain in presence of either glucose or sodium lactate is very greatly inhibited by mapharside and by 3-acetamido-4-carboxyphenylarsenoxide, the inhibitions being greatly reduced or eliminated by the presence of excess cysteine. These results are shown in Table 4 and Figs. 5 and 7. The pentavalent tryparsamide has no inhibitory effect on oxygen uptake by brain in presence of glucose under the conditions of our experiments. We have noted that in all experiments on the respiration of brain tissue in presence of glucose or of lactate, there is an accumulation of a ketonic acid, probably pyruvic acid, in the medium when the trivalent organic arsenical is also present. Doubtless the large inhibitory effects of the trivalent organic arsenicals on brain respiration in presence of glucose or lactate are due partly to the blocking of the respiratory chain at the pyruvic enzyme stage.

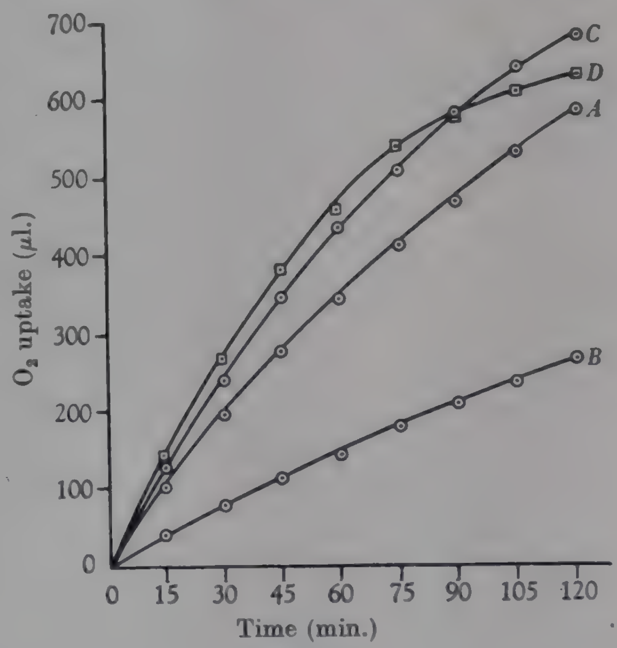


Fig. 5. Effects of mapharside with and without added cysteine, on glucose oxidation at 37°. Exp. 20, Table 4: *A*, glucose oxidation system control; *B*, glucose oxidation system + 170 µg./ml. mapharside; *C*, glucose oxidation system + 3·3 mg./ml. cysteine; *D*, glucose oxidation system + 170 µg./ml. mapharside + 3·3 mg./ml. cysteine.

Lactic dehydrogenase. Results given in Table 4, Exp. 15, show that lactic dehydrogenase, examined by the anaerobic technique, is unaffected by mapharside and is therefore unlikely to be a thiol enzyme.

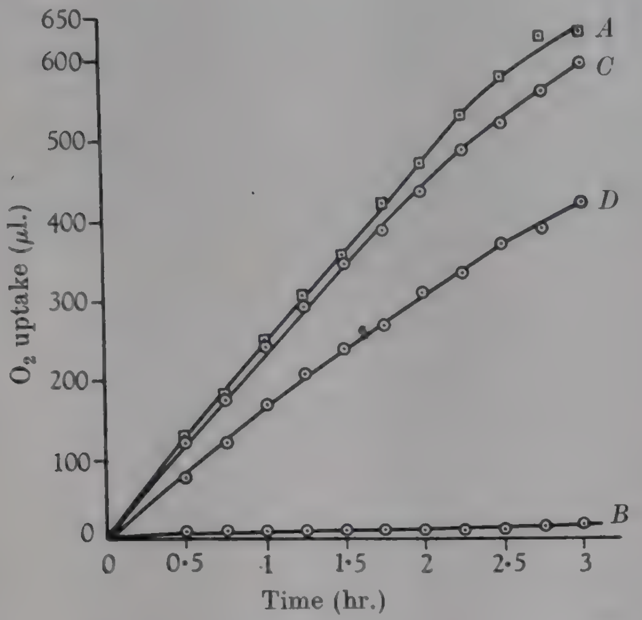


Fig. 6. Effects of mapharside, with and without added glutathione, on pyruvic oxidase activity at 37°. Exp. 23, Table 4: *A*, pyruvic oxidase control; *B*, pyruvic oxidase + 200 µg./ml. mapharside; *C*, pyruvic oxidase + 5 mg./ml. glutathione; *D*, pyruvic oxidase + 200 µg./ml. mapharside + 5 mg./ml. glutathione.

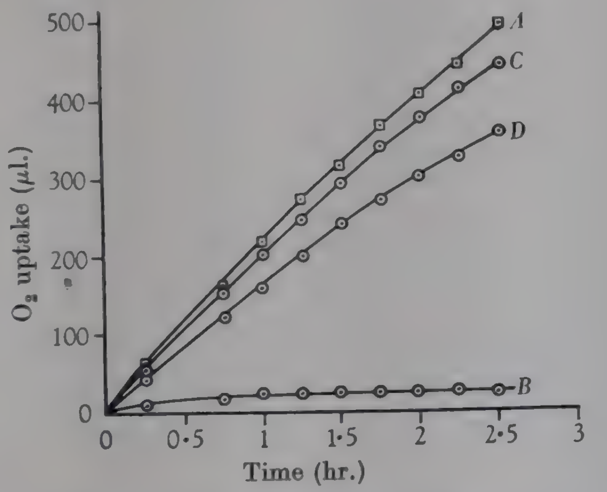


Fig. 7. Effects of mapharside and glutathione on lactic oxidation system at 37°. Exp. 26, Table 4: *A*, lactate oxidation system control; *B*, lactate oxidation system + 17 µg./ml. mapharside; *C*, lactate oxidation system + 330 µg./ml. glutathione; *D*, lactate oxidation system + 17 µg./ml. mapharside + 330 µg./ml. glutathione.

Cytochrome oxidase and catalase. Neither of these enzymes is affected by mapharside under the conditions of our experiments and it is unlikely that they are thiol enzymes.

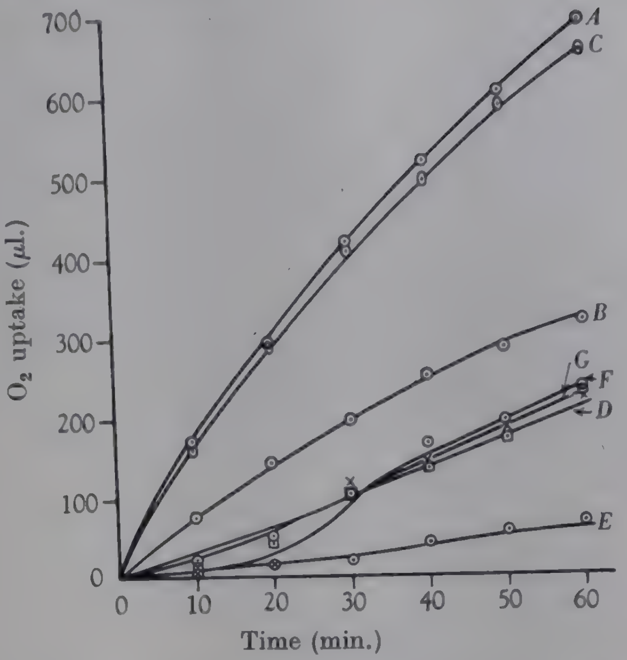


Fig. 8. Effects of arsenic compounds, with and without added cysteine, on choline dehydrogenase activity at 37°. Exp. 9, Table 4: *A*, choline dehydrogenase control; *B*, choline dehydrogenase + 230 µg./ml. reduced tryparsamide thioglycollate; *C*, choline dehydrogenase + 1300 µg./ml. tryparsamide. Exp. 10, Table 4: *D*, choline dehydrogenase control; *E*, choline dehydrogenase + 200 µg./ml. mapharside; *F*, choline dehydrogenase + 200 µg./ml. mapharside + 5 mg./ml. cysteine (cysteine added at same time as mapharside); *G*, choline dehydrogenase + 200 µg./ml. mapharside + 5 mg./ml. cysteine (cysteine added after 2 hr. incubation of enzyme and mapharside).

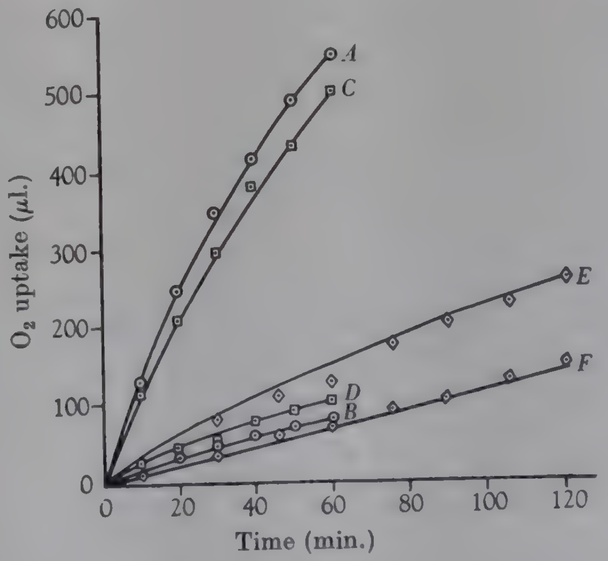


Fig. 9. Effects of thiol inhibitors on choline dehydrogenase activity at 37°. Exp. 2, Table 5: *A*, choline dehydrogenase control; *B*, choline dehydrogenase + 6×10^{-4} M-sodium tetrathionate. Exp. 4, Table 5: *C*, choline dehydrogenase control; *D*, choline dehydrogenase + 0·022 M-alloxan. Exp. 5, Table 5: *E*, choline dehydrogenase control; *F*, choline dehydrogenase + 0·05 M-oxidized glutathione.

Protection of enzymes by their substrates

Experiments were carried out on a few of the enzymes, which were shown to be inhibited by arsenoxides, to determine whether the enzymes could be protected from inactivation by their substrates. The enzymes which were examined in this way were urease, succinic dehydrogenase and choline dehydrogenase. The activities of the enzymes were measured in the manner already described, except that the inhibitor was added before the substrate. Controls were also performed, in which the substrate was added last. Results are shown in Table 6.

Table 6. *Effects of the presence of substrates on inactivation of enzymes by mapharside*

(A) Urease (final concentration of urea 16 %)

Final concentration of mapharside (μg./ml.)	Conditions of experiment	CO_2 output (μl.)	Inhibition of enzyme added (%)
0	Urea added last	1749	—
33	Urea added last	960	45
0	Urea added before mapharside	1791	—
33	Urea added before mapharside	1131	37

(B) Succinic dehydrogenase (conc. of substrate M/30)

Source of enzyme	Wt. of wet tissue (mg.)	Conc. of mapharside (μg./ml.)	Conditions of experiment	O_2 uptake (μl.)	Inhibition of activity (%)
Rat kidney slices	100	0	Succinate added last	393	—
	100	33	Succinate added last	225	44
	100	0	Succinate added before mapharside	399	—
	100	33	Succinate added before mapharside	327	19
Pigeon kidney slices	100	0	Succinate added last	334	—
	100	33	Succinate added last	204	39
	100	0	Succinate added before mapharside	322	—
	100	33	Succinate added before mapharside	282	13
			(C) Choline dehydrogenase		
Rat liver extract (1 in 4)	1 ml. extract	0	—	368	—
	1 ml. extract	133	Choline added last	194	48
	1 ml. extract	133	Choline added before mapharside	281	32

Urease is obviously not protected by its substrate (see also Quastel, 1932). Succinic dehydrogenase is protected to some extent by succinate when the concentration of mapharside is low, and choline dehydrogenase is also partially protected by its substrate.

Reduction of tryparsamide and atoxyl by thiol compounds and the effects of the reduction products on urease

It is already known (Levaditi, Andrews & Mann, 1928) that atoxyl in presence of glutathione and of tissue extracts becomes strongly trypanocidal, an effect attributed to the reduction of the pentavalent arsenical, by thiol compounds, to the active trivalent arsenoxide. This phenomenon has been confirmed with a variety of organic pentavalent arsenic compounds including tryparsamide in the presence of glutathione or whole blood (Hawking & Quastel, 1936).

It became of interest to see whether the reduction products of tryparsamide and atoxyl, due to incubation with thiol compounds, would inactivate urease.

The arsenical (0·1 g.) was dissolved in 5 ml. water to which 10 mg. cysteine was added. The solution was neutralized, placed in a vacuum tube from which air was withdrawn, and the tube was incubated at 37°. The tube was then opened and various quantities of the solutions were added to urease in a Warburg manometric vessel, from the side tube of which urea was tipped after thermal equilibrium.

Typical results are shown in Table 7, which also shows the effects obtained when excess of the thiol compound was added to the urease system. The results show that both atoxyl and tryparsamide may be transformed into highly toxic products by incubation with glutathione, cysteine or thioglycollate. Moreover the toxicities are diminished or eliminated by having an excess of glutathione present with the urease. The facts indicate that urease may be used to detect the formation of trivalent arsenoxide by reduction of pentavalent arsenicals by thiol compounds.

Table 7. *Inhibition of urease activity by pentavalent organic arsenic compounds incubated with thiol compounds*

Exp.	Nature and conditions of incubation mixture	Amount of incubation mixture added to urease (ml.)	Duration of exp. with urease (min.)	CO_2 output (μl.)	Inhibition of urease activity (%)
1	—	Nil	35	612	—
	Atoxyl (20 mg./ml.) + cysteine (2 mg./ml.) incubated 72 hr. at 37°	1	35	44	93
2	—	Nil	40	717	—
	Atoxyl (20 mg./ml.) + cysteine (2 mg./ml.) incubated 72 hr. at 37°	0·5	40	104	86
	Tryparsamide (20 mg./ml.) + cysteine (2 mg./ml.) incubated 72 hr. at 37°	0·5	40	81	89
3	—	Nil	40	471	—
	Tryparsamide (20 mg./ml.) + Na thioglycollate (2 mg./ml.) incubated 19 hr. at 37°	0·1	40	167	65
	Tryparsamide (20 mg./ml.) + cysteine (2 mg./ml.) incubated 19 hr. at 37°. Excess glutathione (10 mg.) finally added on transference to urease	0·1	40	377	20
4	—	Nil	40	640	—
	Tryparsamide (20 mg./ml.) + cysteine (2 mg./ml.) incubated 19 hr. at 37°	0·1	40	167	74
	Tryparsamide (20 mg./ml.) + cysteine (2 mg./ml.) incubated 19 hr. at 37°. Excess glutathione (10 mg.) added on transference to urease	0·1	40	547	7
5	—	Nil	30	457	—
	Tryparsamide (20 mg./ml.) + glutathione (2 mg./ml.) incubated 20 hr. at 37°	0·5	30	271	40
	Tryparsamide (20 mg./ml.) + glutathione (2 mg./ml.) incubated 20 hr. at 37°. Excess glutathione (5 mg.) added on transference to urease	0·5	30	384	16
6	—	Nil	50	323	—
	Tryparsamide (20 mg./ml.) + glutathione (2 mg./ml.) incubated 20 hr. at 37°	0·5	50	195	40

DISCUSSION

Our experimental results justify the view that trivalent organic arsenoxides may be employed for the detection of thiol enzyme systems. Four enzymes, namely urease, succinic dehydrogenase, cholinesterase, and pyruvic oxidase, for which there was prior evidence that thiol groups are important for their activities, are highly sensitive to phenylarsenoxide and its derivatives. Enzymes, however, for which there has been no evidence that thiol groups are essential for their activities, viz. lactic dehydrogenase, cytochrome oxidase, catalase and invertase, are relatively unaffected by the presence of organic arsenoxides.

Pentavalent organic arsenic compounds such as tryparsamide or atoxyl do not affect enzymes (except in the case of liver esterase), but they become active poisons to the thiol enzymes when they are reduced to the trivalent arsenoxides by the presence of thiol compounds.

The organic arsenoxides probably establish an equilibrium with the thiol enzymes, as their toxicities do not increase with time. These toxicities may be diminished or eliminated by the presence of an excess of thiol compounds which convert the arsenoxides into inert condensation products whose dissociations are smaller the greater the excess of free thiol groups.

Choline dehydrogenase is shown, by its high sensitivity to organic arsenoxide, or to the condensation products of these substances with thioglycollic acid, which dissociate freely in absence of excess thiol compounds, to be a thiol enzyme. This is confirmed by showing the enzyme to be sensitive also to the presence of sodium tetrathionate, alloxan or oxidized glutathione, molecules which bring about the oxidation of SH groups. This enzyme, too, probably forms an equilibrium with organic arsenoxide, and the enzyme inactivated by this means may be reactivated by the presence of excess thiol groups, as is also the case with the other thiol enzymes investigated (succinic dehydrogenase, urease, pyruvic oxidase, cholinesterase).

It is obvious that these results have a bearing on the therapeutic properties of organic arsenicals. The reduction of these substances by tissue constituents and the removal of the resultant arsenoxides by

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combination with inert proteins, or their transformation into inert condensation products by local high concentrations of SH bodies, are important factors to be considered in the problems of arsenic therapy.

SUMMARY

1. A description is given of experiments carried out during 1938, 1939 and early 1940, details of which have not hitherto been published, on the effects of organic trivalent and pentavalent arsenic compounds on enzyme systems.

2. Urease is highly sensitive to trivalent arsenoxides such as phenylarsenoxide, *m*-amino-*p*-hydroxyphenylarsenoxide (mapharside), *p*-acetamidophenylarsenoxide, and *m*-acetamido-*p*-carboxyphenylarsenoxide. It is also inhibited by the products of condensation of thioglycollate with phenylarsenoxide and with the reduced forms of atoxyl and tryparsamide. The effects are generally greater than those due to sodium arsenite at equivalent concentrations. Urease is also sensitive to stabilarsan, the diglucoside of arsphenamine, but this substance is less toxic than sodium arsenite at equivalent concentrations. The toxicity of arsenoxides may be reduced or eliminated by the presence of excess of thiol compounds such as cysteine or glutathione, or by the presence of proteins which compete with urease for the arsenoxides. An equilibrium probably exists between the arsenoxide and the enzyme. Pentavalent arsenic compounds such as atoxyl or tryparsamide show little toxicity to urease. The results conform with the view that urease is a thiol enzyme.

3. The reaction of cholinesterase with arsenicals also supports the conclusion that this enzyme is a thiol enzyme.

4. Choline dehydrogenase is shown to be a thiol enzyme, since it is inhibited by trivalent arsenoxides, the inhibition being neutralized by the presence of excess of thiol compounds. It is also inhibited by sodium tetrathionate, alloxan, and oxidized glutathione.

5. Pyruvic oxidase is greatly inhibited by mapharside, the inhibition being suppressed by the presence of excess of glutathione. These results point to the thiol nature of the enzyme. The oxidation systems of lactate and of glucose, in brain tissue, are also inhibited by trivalent arsenoxide, the effects being reversed by the presence of excess thiol compounds. Lactic dehydrogenase, however, is unaffected by mapharside and is therefore unlikely to be a thiol enzyme.

6. Cytochrome oxidase, catalase and invertase are relatively unaffected by the presence of organic arsenoxides.

7. Pentavalent organic arsenic compounds do not affect the enzymes investigated, except in the case of liver esterase, but they become active poisons to the thiol enzymes when they are reduced to the trivalent arsenoxides by the presence of thiol compounds.

8. The thiol enzymes apparently form reversible equilibria with organic trivalent arsenoxides.

9. Whereas succinate protects its enzyme from inactivation by a trivalent arsenoxide and choline likewise shows some protection to its enzyme, urea fails to afford any protection to urease.

10. Mapharside is recommended as a useful organic trivalent arsenical for the detection of thiol enzymes.

We are greatly indebted to Dr H. King, F.R.S., for his gift of organic trivalent arsenoxides and their condensation products with thioglycollic acid.

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The Metabolism of Sulphonamides

5. A STUDY OF THE OXIDATION AND ACETYLATION OF SULPHONAMIDE DRUGS AND RELATED COMPOUNDS IN THE RABBIT

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It has now been established that both the simple and heterocyclic sulphonamides may undergo both acetylation and oxidation in the body (cf. Williams, 1945, 1946, 1947; Dobson & Williams, 1946; Stubbs & Williams, 1947; Bray, Neale & Thorpe, 1946; Gilligan, 1945; Scudi & Silber, 1944; Weber, Lalich & Major, 1943). In the simpler sulphonamides, such as the *o*-, *m*- and *p*-aminobenzenesulphonamides, oxidation occurs in the benzene ring, whereas in the *N'*-substituted sulphonamides, sulphapyridine and sulphaquinoxaline (Bray *et al.* 1946; Weber *et al.* 1943; Scudi & Silber, 1944), oxidation occurs in the heterocyclic ring and acetylation in the benzene ring.

The object of the present work was to find out whether *in vivo* oxidation and acetylation were related. These two processes could occur in four ways: (1) acetylation followed by oxidation of the acetylated molecule, (2) oxidation followed by acetylation of the oxidized molecule, (3) simultaneous oxidation and acetylation of the same molecule, and (4) oxidation and acetylation as separate reactions involving different molecules of the compound:

(1) $R\mathrm{H.NH_2} \rightarrow R\mathrm{H.NHAc} \rightarrow R\mathrm{(OH)NHAc}$.
(2) $R\mathrm{H.NH_2} \rightarrow R\mathrm{(OH)NH_2} \rightarrow R\mathrm{(OH)NHAc}$.
(3) $R\mathrm{H.NH_2} \rightarrow R\mathrm{(OH)NHAc}$.
(4) $2R\mathrm{H.NH_2} \rightarrow R\mathrm{H.NHAc} + R\mathrm{(OH)NH_2}$.

It was suggested by Dobson & Williams (1946) that, in the case of metanilamide, oxidation does not occur in the molecule undergoing acetylation *in vivo*. Furthermore, orthanilamide is highly oxidized but hardly acetylated (Stubbs & Williams, 1947), whereas sulphanilamide is highly acetylated but only slightly oxidized in the rabbit (Shelswell & Williams, 1940; Williams, 1945, 1946). There are, therefore, suggestions that the fourth of the above mechanisms is plausible.

In the present work evidence that the compound studied has been oxidized is based on glucuronic acid and ethereal-sulphate determinations. If a compound devoid of hydroxyl groups is fed to an animal and gives rise to the excretion of increased amounts of glucuronic acid and/or ethereal sulphate, it is reasonable to conclude that the compound has been oxidized. One has, however, to bear in mind that oxidation could take place without the formation of *O*-conjugates and in such cases proof of oxidation requires the isolation of the oxidation product (cf. Ambamide; Hartles & Williams, 1947). Such cases, however, are likely to be uncommon in the series of compounds concerned here.

METHODS

Animals

Rabbits (giant chinchilla) were used throughout this investigation. They were maintained on a constant diet of 50 g. of Lever's cubes, with water *ad lib.* All drugs were administered with water by stomach tube.

Analytical methods

Glucuronic acid in urine was determined by the method of Hanson, Mills & Williams (1944). Sanchez (1945) has observed a colour reaction between furfural and sulphadiazine. This fact had to be considered when glucuronic acid was determined in the presence of pyrimidine compounds. Glucuronic acid was therefore estimated in pure glucurone solutions in the presence of sulphadiazine and sulphapyrazine and their derivatives. None of these drugs had any influence on the recovery of glucuronic acid.

Ethereal sulphate was determined by the Folin gravimetric method (cf. Williams, 1938).

Free and combined diazotizable amino groups were determined by the colorimetric method of Bratton & Marshall (1939) using *N*-(1-naphthyl)-ethylenediamine as coupling agent. Colour intensities were measured by means of a Spekker photoelectric absorptiometer with suitable filters (in most cases Ilford no. 604 green or no. 605 yellow green). Standard curves were constructed for each drug studied.

Free amino groups were determined directly on urine appropriately diluted. Total amino groups were determined after acid hydrolysis. The concentration of acid and duration of hydrolysis were varied to suit the compound under investigation. The extent of acetylation was calculated as the difference between the values for free and total diazotizable amino groups. This calculation, however, is not valid in every case, particularly in those cases where oxidation is extensive (see Dobson & Williams, 1946).

RESULTS

Sulphadiazine, sulphapyrazine and their derivatives

The collected results for these compounds are given in Table 1. In Table 2 a complete set of results is given for sulphamezathine in order to illustrate the type of data on which the average figures in Table 1 and other tables are based.

Table 1. *The oxidation and acetylation of sulphadiazine and sulphapyrazine and their derivatives in the rabbit*

		Drug excreted					
		Total	Free	Acetylated		Oxidized (% of dose)	
Compound fed	Dose (g./kg.)	(% of dose)		(% of dose)	(% of total)	Glucuronide	Ethereal sulphate
Free drugs:							
Sulphadiazine	0·2	74	16	58	78	—	0
	0·5	82	26	56	68	5	0
	1·0	92	21	72	78	0	—
Sulphamerazine	0·2	76	7	69	91	—	0
	0·5	81	9	72	89	8	0
Sulphamezathine	0·2	87	10	77	88	15	0
	0·5	90	16	74	82	16	0
Sulphapyrazine	0·2	74	10	64	86	—	—
	0·5	83	10	73	88	0	—
Acetylated drugs:							
Acetylsulphadiazine	0·45	75	0·5	75	100	0	—
Acetylsulphamerazine	0·4	71	0·5	71	100	0	—
Acetylsulphamezathine	0·5	102	0·5	102	100	0	—
Acetylsulphapyrazine	0·5	79	1·0	78	99	0	—
Oxidized drugs:							
SDOH*	0·2	90	78	12	13	0	—
SM.COOH	0·2	81	73	8	10	0	—
AcSM.COOH	0·2	82	1	81	99	0	—

* SDOH is 2-sulphanilamido-4-hydroxypyrimidine (hydroxysulphadiazine); SM.COOH is 2-sulphanilamido-4-carboxy-6-methylpyrimidine; AcSM.COOH is 2-(N^4-acetylsulphanilamido)-4-carboxy-6-methylpyrimidine.

Table 2. *The excretion of metabolites of sulphamezathine in the rabbit*

(Dose 0·5 g./kg.)

	Amount of drug recovered (% of dose)				
Rabbit no.	Total	Free	Acetylated	As glucuronide*	As ethereal sulphate
72	64	8	56	—	—
68	86	21	65	—	—
71	96	15	81	15	—
78	94	16	78	14	—
76	91	14	76	10	—
67	93	9	84	16	—
78	98	21	77	12	—
81	95	22	72	16	—
60	—	—	—	—	0
66	—	—	—	—	0
67	—	—	—	—	0

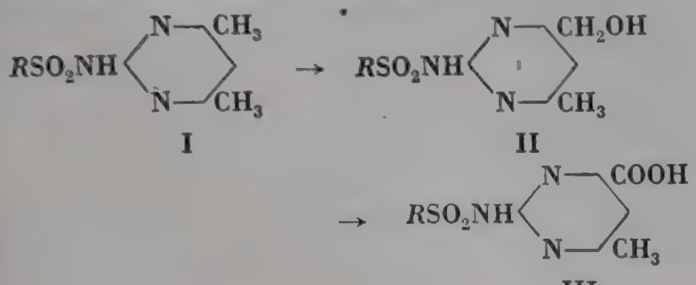
* It is assumed that one molecule of the drug gives rise to one molecule of glucuronic acid.

Table 1 shows that neither sulphadiazine (m.p. 252°) nor sulphapyrazine (m.p. 259°) undergoes any oxidation in the rabbit, whereas the substituted sulphadiazines, sulphamerazine (m.p. 236°) and sulphamezathine (m.p. 198°) both give rise to increased outputs of glucuronic acid. None of these compounds gives rise to ethereal sulphates. Similar results were obtained by Gilligan (1945) on hospital patients undergoing treatment with these four drugs. Some of Gilligan's results indicate an oxidation up to 68 % with sulphamezathine, but none at all with sulphadiazine and sulphapyrazine.

All four compounds are highly acetylated, although sulphadiazine is less acetylated than the other three. When the acetylated drugs are fed they are excreted unchanged, being neither deacetylated nor oxidized. Previous work on N^4-acetylsulphonamides had shown that they were not deacetylated in man (e.g. Strauss, Lowell, Taylor & Finland, 1941).

A comparison of the figures (in Table 1) for the amounts of sulphamerazine and sulphamezathine excreted free, with those for the amounts oxidized, shows that they correspond. These figures can be interpreted as meaning that it is the free and not the acetylated drugs that are oxidized. This interpretation is supported by the finding that neither N^4-acetylsulphamerazine nor N^4-acetylsulphamezathine is oxidized. These results indicate that oxidation and acetylation are separate reactions involving different molecules of the drug. If, therefore, acetylation of these two drugs could be suppressed, the extent of their oxidation would be expected to increase.

Several unsuccessful attempts were made to isolate and identify the glucuronide in sulphamezathine urine. Nevertheless, a consideration of Table 1 does allow us to make a suggestion concerning the possible nature of this oxidation product. The main difference between sulphadiazine on the one hand, and sulphamerazine and sulphamezathine on the other, lies in the presence of substituent methyl groups in the pyrimidine rings of the latter. It is possible that these methyl groups may undergo oxidation. In the case of sulphamezathine (I) oxidation could proceed as follows:

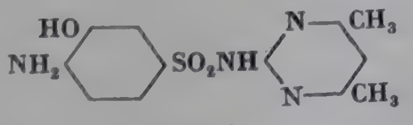


I II III

(where $R = NH_2C_6H_4$—).

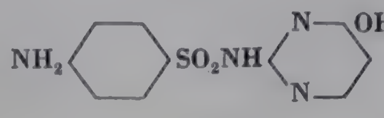
Compound (III) (2-sulphanilamido-6-methylpyrimidine-4-carboxylic acid, m.p. 253°) was therefore prepared (see below) and fed to rabbits. This compound, however, did not form a glucuronide and was only slightly acetylated (8 %; see Table 1). Its N^4-acetyl derivative, m.p. 260° (prepared according to Rose & Swain, 1945), like other acetylated sulphonamides, is excreted unchanged. If this acid formed a glucuronide it would be expected to be an ester glucuronide, i.e. a substance which would reduce Fehling's solution (cf. benzoylglucuronide). Sulphamezathine urine, however, does not reduce Fehling's solution, and is therefore unlikely to contain a glucuronide of this type. The carboxylic acid (III) forms a characteristically insoluble copper salt (see below), but such a copper salt could not be isolated from sulphamezathine urine appropriately treated.

Another possible oxidation product of sulphamezathine is 2-(3′-hydroxysulphanilamido)-4:6-dimethylpyrimidine (IV), the analogue of 3-hydroxysulphanilamide (cf. Williams, 1946, 1947). Such a compound, however, would be expected to form an ethereal sulphate. But there is no detectable increase in ethereal sulphate output after feeding sulphamezathine. Compound (IV) is therefore a possible but not probable oxidation product. Since sulphamezathine gives rise to a non-reducing glucuronide with no ethereal sulphate, a compound such as 2-sulphanilamido-4-hydroxymethyl-6-methylpyrimidine (II) would be an oxidation product which would fit the evidence obtained so far. We have not been able to synthesize this compound.

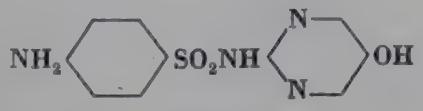


IV

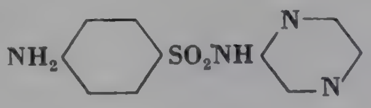
Table 2 also includes 4-hydroxysulphadiazine (V). Theoretical considerations of the electron distribution on the pyrimidine ring indicate that position 5 is the most likely point of attack. If sulphadiazine were oxidized *in vivo* then 5-hydroxysulphadiazine (VI) would be expected to be formed. We have not synthesized (VI), but we prepared (V), m.p. 280°, according to Ganapathi, Deliwala & Shirsat (1942). On feeding (V) to rabbits it was largely excreted unchanged although some 12 % was acetylated. It formed no glucuronide. We were unable to detect any hydroxysulphadiazine in sulphadiazine urine. The only compound isolated was N^4-acetylsulphadiazine (m.p. and mixed m.p. 245°), which was obtained in excellent yields by continuous ether extraction of sulphadiazine urine brought to pH 1 with HCl. N^4-Acetylsulphamerazine (m.p. and mixed m.p. 242°) and N^4-acetylsulphamezathine (m.p. and mixed m.p. 241°) were isolated from the corresponding urines in a similar manner.



V



VI



VII

Sulphapyrazine (VII) undergoes no oxidation in the rabbit, although it is highly acetylated (88 %). Biological oxidation in the pyrazine ring is unlikely on theoretical grounds, for positions 3, 5 and 6 are all deactivated by the ring nitrogens (cf. Krems & Spoerri, 1947).

Synthesis of 2-sulphanilamido-6-methylpyrimidine-4-carboxylic acid. The acetyl derivative of this acid was prepared according to Rose & Swain (1945). The acetyl compound (4·5 g.) was boiled for 1 hr. with 50 ml. 5N-KOH. The mixture was treated with charcoal and filtered. The hot solution was treated with dilute H_2SO_4 till strongly acid to litmus and the *carboxylic acid* separated as a yellow precipitate. After cooling to 0° the crystalline solid was filtered off. It was sparingly soluble or insoluble in water and the usual organic solvents, and was purified by dissolving in hot alkali and precipitating with H_2SO_4. It formed pale-yellow short needles, m.p. 253–256°, and titrated as a dibasic acid. (Found: N, 18·3; equiv. by titration, 157. $C_{12}H_{12}O_4N_4S$ requires N, 18·2%; equiv. 154.) (Ultraviolet absorption spectrum: one band at $\lambda_{\text{max.}}$ 255 mμ. with $\epsilon_{\text{max.}}$ 19,400.) It forms an insoluble copper salt. This salt was prepared by dissolving the acid in dilute NaOH and making just acid with dilute HCl to give a supersaturated solution, and then adding $CuSO_4$ solution. The salt was precipitated as a pale-blue micro-crystalline powder insoluble in water, dilute HCl and acetic acid, but dissolving in cold KOH and conc. HCl. No precipitates were obtained on adding soluble Zn, Ba, Ca, Hg, Pb and Fe^{+++} salts to a supersaturated solution of the acid. The Ag salt was insoluble in water.

Sulphathiazole, sulphapyridine and sulphaguanidine

The results for these compounds and their N^4-acetyl derivatives are given in Table 3.

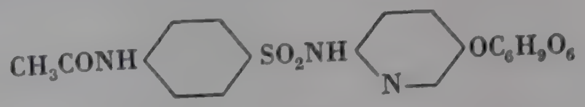
Table 3. *The oxidation and acetylation of sulphathiazole, sulphapyridine and sulphaguanidine in the rabbit*

Compound fed	Dose (g./kg.)	Total (% of dose)	Free (% of dose)	Acetylated		Oxidized as glucuronide (% of dose)
				(% of dose)	(% of total)	
Free drugs:						
Sulphathiazole	0·3	51	30	21	41	3
	0·45	82	31	51	62	8
	1·0	92	33	59	64	6
Sulphapyridine	0·75	67	19	48	72	29
Sulphaguanidine	0·2	60	7	53	88	?*
Acetyl derivatives:						
Acetylsulphathiazole	1·0	8	0·5	7·5	94	0
Acetylsulphapyridine	0·75	62	1	61	98	28
Acetylsulphaguanidine	0·2	63	6	56	89	?*

* These drugs are too slowly absorbed to allow glucuronic acid determinations of any significance.

Sulphathiazole (m.p. 195°) is less highly acetylated than the compounds of the sulphadiazine series. It does, however, undergo a small but significant oxidation of about 6% of the dose. Its acetyl compound is very poorly absorbed, and after several days only about 8% of the dose is excreted in the urine. The deacetylation of this compound is not significant.

In the case of sulphapyridine (m.p. 196°) we have an example of a sulphonamide in which oxidation and acetylation may occur in the same molecule. The figures in Table 3 indicate that this may be a case of a compound which is first acetylated and then oxidized, for the oxidation of N^4-acetylsulphapyridine (m.p. 230°) occurs to the same extent (28%) as with sulphapyridine itself (29%). Although the acetyl compound is oxidized it does not undergo significant deacetylation. Other workers (Weber *et al.* 1943; Bray *et al.* 1946) have shown that sulphapyridine is oxidized somewhere in the heterocyclic ring. The present work indicates that the glucuronide occurring in rabbit urine is likely to be partly acetylated. Considerations of the electron distribution on the pyridine ring suggest that the β-positions are the most likely to be oxidized, and therefore a glucuronide of sulphapyridine can be tentatively formulated as 2-(*p*-acetylsulphanilamido)-3-or-5-glucuronosidopyridine (VIII is the 5-glucuronide).



VIII

In the case of sulphaguanidine (m.p. 186°) and its acetyl derivative (m.p. 260°) absorption is so slow that glucuronide formation, if it occurred, could not be detected. Sulphaguanidine itself is highly acetylated and its acetyl derivative appears to undergo a slight deacetylation, possibly by the organisms of the gut.

Sulphanilic and p*-aminobenzoic acids*

The results for these compounds and their acetyl derivatives are given in Table 4. Sulphanilic acid is excreted unchanged, there being no acetylation and no oxidation. Acetylsulphanilic acid behaves similarly, there being no deacetylation and no oxidation. These results are what would be expected, for both compounds are comparatively strong acids and the body rapidly excretes strong acids unchanged (cf. Quick, 1932*b*). Hildebrandt (1906) has reported that sulphanilic acid is excreted unchanged by dogs, but Zehender (1943) reports that it is highly acetylated in guinea pigs. *p*-Aminobenzoic acid is partly excreted unchanged, partly acetylated (20%) (cf. Harrow, Mazur & Sherwin, 1933) and partly conjugated with glucuronic acid to form an ester glucuronide (15%). This glucuronide is probably largely *p*-aminobenzoylglucuronide for *p*-acetamidobenzoic acid forms a negligible amount (4%) of glucuronide and is mainly excreted unchanged.

Table 4. *The oxidation and acetylation of sulphanilic and* p-*aminobenzoic acid in the rabbit*

Compound	Dose (g./kg.)	Total (% of dose)	Free (% of dose)	Acetylated (% of dose)	Acetylated (% of total)	Oxidized (% of dose) As glucuronide	Oxidized (% of dose) Ethereal sulphate
Sulphanilic acid	0·63	97	95	0	0	0	—
p-Aminobenzoic acid	0·4	94	72	22	23	0 (15)*	0
Acetylsulphanilic acid	0·5	99	1	98	99	0	—
p-Acetamidobenzoic acid	0·4	90	0·3	90	100	0 (4)*	—
p-Acetamidophenylacetic acid	0·5	55	0·2	55	100	—	—

* As ester glucuronide (see text).

p-Aminobenzoic acid does not undergo nuclear oxidation for it does not give rise to an ethereal sulphate. It could give rise to a glycine conjugate, but we did not look for it since Quick (1932*a*) has reported that it does not form a glycine conjugate in dogs. The work of Smith, Bayliss, Orgorzalek & McClure (1946), however, suggests that *p*-aminohippuric acid may be formed in man.

p-Acetamidophenylacetic acid (m.p. 168°) was included in this series because we wished to find out whether removal of the carboxyl group from the ring would influence the *in vivo* stability of the *p*-acetamido group. Table 4 shows that this acid is not deacetylated in the rabbit.

DISCUSSION

All the compounds studied here are derivatives of aniline in which there is a para-substituted acidic group (aniline substituted with other types of groups will be the subject of another paper). It is therefore interesting to compare the acid dissociation constants of these compounds with the extent of their acetylation in the rabbit (see Table 5).

Table 5. *The relation between dissociation constant and extent of acetylation of certain* p-*substituted anilines*

(pK_a's quoted from Bell & Roblin (1942).)

Compound	pK_a	Drug excreted as acetyl compound (average figures) (%)
Sulphanilic acid	3·24	0
p-Aminobenzoic acid	4·68	23
Sulphapyrazine	6·04	87
Sulphadiazine	6·48	72
Sulphamerazine	7·06	90
Sulphathiazole	7·12	56
Sulphamezathine	7·37	85
Sulphapyridine	8·43	72
Sulphanilamide	10·43	50

It appears from this table that there is a rough relationship between pK_a and percentage acetylation. When pK_a is small, i.e. the compound is a relatively strong acid, acetylation only takes place to small extent, e.g. sulphanilic acid, $pK_a = 3·24$ and acetylation 0%; *p*-aminobenzoic acid, $pK_a = 4·68$ and acetylation, 23%. Compounds with pK_a's varying from about 6 to 9 are all highly acetylated. It appears probable from other work carried out in this laboratory that when pK_a is very large, i.e. the compound is not an acid or is an extremely weak acid, then the percentage acetylation again becomes very small (e.g. aniline, unpublished results). These facts suggest that there is an optimum extent of ionization for biological acetylation. If the compound is too highly ionized either as an acid or a base, there is a tendency for acetylation to be low or zero. The alternative explanation is that if a compound is strongly acidic there is a tendency for rapid excretion and there is little time for acetylation. We found, for example, that sulphanilic acid was rapidly excreted by the rabbit.

Another fact which emerges from the present results is that acetylation of the aromatic amino group is a one way reaction. None of the acetamido compounds studied here were deacetylated by the rabbit to any significant extent. Other work in this laboratory indicates that this is true also for such compounds as acetanilide, phenacetin, *p*-acetamidophenol, etc. According to Lipmann (1946) the acetylation of the aromatic amino group in sulphanilamide involves the participation of adenosine triphosphate and acetate, and since the reaction is highly exergonic, the tendency is complete acetylation. Reversal of this reaction resulting in deacetylation is therefore unlikely. However, deacetylation could be brought about by hydrolysis, which is not the reverse of acetylation, by the mechanism suggested by Lipmann. Michel, Bernheim & Bernheim (1937) and Kohl & Flynn (1940) have reported that rat liver and kidney contains an acylase which deacetylates acetylated sulphonamides and acetanilide *in vitro*. In the intact rabbit, if this enzyme system does occur, there appear to be considerable limitations to its activity.

The present work also gives us some information on the relation of oxidation to acetylation. It appears, as far as the compounds studied here are concerned, that there are two types of amino compounds involved, (1) those in which oxidation and acetylation require separate molecules one for each process, e.g. sulphamezathine, and (2) those in which oxidation and acetylation can occur in the same molecule, e.g. sulphapyridine (cf. also β-naphthylamine, Dobriner, Hofmann & Rhoads, 1941; and 2-acetamidofluorene, Bielschowsky, 1945). Other types of aromatic compounds, such as those which are oxidized but not acetylated, are also known and will be discussed in another communication.

SUMMARY

The extent of acetylation and of oxidation in the rabbit of certain *N*-substituted heterocyclic sulphonamide drugs has been measured. Similar measurements have been made on sulphanilic acid, *p*-aminobenzoic acid and related compounds. The following conclusions were reached:

(1) All sulphonamides with pK_a's between 6 and 9 are highly acetylated; compounds with a pK_a less than 6 were not highly acetylated.

(2) *p*-Substituted acetanilides are not deacetylated by the rabbit.

(3) In some sulphonamides, e.g. sulphamerazine, sulphamezathine and sulphathiazole, oxidation occurs in the free drug but not with the acetylated drug, whereas in sulphapyridine the acetyl derivative and the free drug are oxidized.

(4) Sulphadiazine and sulphapyrazine are not oxidized.

(5) Sulphanilic acid is neither acetylated nor oxidized whereas *p*-aminobenzoic acid is slightly acetylated (*c.* 20 %) but not oxidized.

(6) The results obtained have been related as far as possible to certain physicochemical concepts.

We wish to acknowledge our gratitude to May and Baker Ltd. for gifts of sulphadiazine, sulphapyrazine, sulphathiazole and sulphapyridine, to Imperial Chemical Industries (Pharmaceuticals) Ltd. for sulphamezathine and sulphaguanidine and to Sharpe and Dohme Ltd. for sulphamerazine. The expenses of this work were in part defrayed by a grant from the Medical Research Council.

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Studies in Detoxication

15. ON THE GLUCURONIDES OF STILBOESTROL, HEXOESTROL AND DIENOESTROL

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The importance of stilboestrol, hexoestrol and dienoestrol as chemotherapeutic substances of value as oestrogens and as anticancer compounds has now been established (cf. Dodds, 1945; Haddow, 1947). Their fate in the body, however, has not been fully worked out. In 1939 Stroud (1939*a*) showed that stilboestrol, on injection into rabbits, was excreted both in a free and combined state and, in 1942, Mazur & Shorr isolated stilboestrol monoglucuronide from the urine of rabbits receiving small doses of stilboestrol by injection. The glucuronide isolated corresponded to 30% of the dose. Since stilboestrol is a phenol it would be expected to form an ethereal sulphate. Teague (1945), however, has reported that it causes no significant change in the organic sulphate output of rabbits or rats. The same worker found no change in the neutral sulphur output. These experiments with intact animals therefore suggest that stilboestrol undergoes but one change in the body, namely conjugation of one of its hydroxyl groups with glucuronic acid. *In vitro* experiments carried out by Zimmerberg (1946) (cf. Zondek, Sulman & Sklow, 1943) showed that stilboestrol disappeared when added to rat-liver slices in a Ringer-bicarbonate medium at 37°. The stilboestrol was believed to be conjugated or oxidized, only one hydroxyl being attacked. Zimmerberg suggested that conjugation could take place with sulphuric or another acid, probably glucuronic acid, depending on which is available.

In the case of hexoestrol and dienoestrol Stroud (1939*a*) showed that both these compounds were excreted in a combined form by rabbits. Up to the present time no reliable data have been available on the quantitative aspects of the conjugation of these three oestrogens. In the present paper quantitative determinations of the outputs of glucuronic acid and ethereal sulphate in rabbits following the feeding of these compounds will be presented. A description is also given of the isolation and characterization of the glucuronides. Further work is in progress on these glucuronides, for stereochemical considerations suggest that the nature of the glucuronides from hexoestrol and dienoestrol may be more complicated than is apparent at first sight.

METHODS

Animals. Rabbits (giant chinchilla), kept on a constant diet of 50 g. of Lever's cubes and 100 g. of cabbage *per diem*, were used throughout these experiments. Compounds were administered in all cases in aqueous suspension by means of a stomach tube.

Glucuronic acid was determined according to the method of Hanson, Mills & Williams (1944).

Ethereal sulphate and neutral sulphur were determined gravimetrically by the Folin method (cf. Peters & Van Slyke, 1932).

Spectroscopic observations were made with a Hilger E3 quartz spectrograph.

RESULTS

Experiments with stilboestrol

Quantitative results. These are given in Table 1, and a typical experiment is illustrated in Fig. 1. The results show that at a dose level of 150 mg./kg. an average of 71% of the dose of stilboestrol can be accounted for as a monoglucuronide excreted in the urine. The glucuronic acid output is raised above normal for about 4 days, maximum excretion of the glucuronide occurring on the second day after dosing. Neither the ethereal sulphate nor the neutral sulphur output appears to be disturbed.

Isolation of stilboestrol mono-β-D-glucuronide. Stilboestrol (0·8 g.) (British Drug Houses Ltd., m.p. 173°) was fed to each of 6 rabbits and the urines were collected each day during the next 4 days. Mazur & Shorr (1942) have reported that the sodium salt of stilboestrol glucuronide is relatively insoluble in water. Since rabbit urine is normally alkaline (pH > 8), it is reasonable to expect that the urinary deposit would contain appreciable amounts of this sodium salt. The urines were therefore centrifuged and the deposits obtained worked up separately. The deposits were triturated with small amounts of dilute H_2SO_4, and then anhydrous Na_2SO_4 was added to give a thick pasty mass. The latter was now triturated with ether (5 × 100 ml. portions). The ether extracts were reduced to 100 ml. and the insoluble sodium salt was precipitated on adding 30 ml. saturated $NaHCO_3$ solution. After keeping at 0° for 1–2 hr. the salt was filtered and dried *in vacuo*. The centrifuged urines were acidified with dilute H_2SO_4 and extracted with 4 or 5 portions of ether. The ethereal extracts were then treated as in the case of the deposits. The yields in a good

Table 1. *Excretion of glucuronic acid, ethereal sulphate and neutral sulphur by rabbits receiving stilboestrol orally*

Rabbit no.	Wt. (kg.)	Dose (mg./kg.)	'Extra' glucuronic acid* (% of dose)	'Extra' ethereal sulphate (% of dose)	'Extra' neutral sulphur (% of dose)
81	2·6	150	77·3	—	—
87	2·85	150	73·9	—	—
89	2·75	150	67·1	—	—
90	2·9	150	66·2	—	—
97	2·35	250	—	0	—
69	2·45	250	—	0	0
82	3·0	100	—	0	—
86	2·75	100	—	0	0
80	3·1	250	—	—	0

* Calculated as a monoglucuronide.

experiment are given in Table 2. For analysis the salt (plates) was purified by recrystallization from methanol. (Found: Na, 4·7%; calc. for $C_{24}H_{27}O_8Na$, Na, 4·7%.) To obtain the free glucuronide, the sodium salt was triturated with dilute H_2SO_4, and then anhydrous Na_2SO_4 added to form a thick pasty mass. The glucuronide was extracted from this by stirring with several portions of ether. The ethereal extracts were taken to dryness and the residue dissolved in a small amount of acetone, from which stilboestrol monoglucuronide *dihydrate* crystallizes on adding benzene in excess. On recrystallization from benzene-acetone mixtures it forms masses of long needles, m.p. 179° (decomp.), sintering at 65–75° and showing $[\alpha]_D^{20^\circ} = -56{\cdot}5^\circ$ ($c=1$ in ethanol). (Found: C, 59·8; H, 6·8%; equiv. by titration, 489; glucuronic acid, 39%. $C_{24}H_{28}O_8.2H_2O$ requires C, 60·0; H, 6·7%; equiv. 481; glucuronic acid, 40·3%.) The determination of water of crystallization was not satisfactory since the compound decomposed on prolonged heating at 110°. It was sparingly soluble in cold water and benzene, but readily soluble in ethanol, acetone, ether and hot water. It gave an intense naphthoresorcinol reaction and a deep cherry-red colour in the Dingemanse $SbCl_5$ reaction (see Dingemanse, 1940).

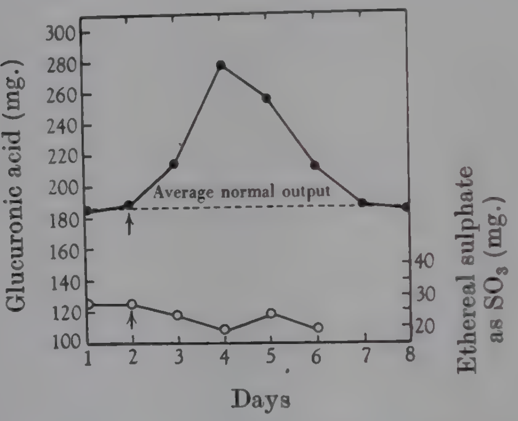

Fig. 1. The glucuronic acid and ethereal sulphate output in rabbits receiving stilboestrol orally. •—•, glucuronic acid curve for rabbit no. 90 that received 440 mg. of stilboestrol on day 2. o—o, ethereal sulphate output in rabbit no. 86 that received 275 mg. stilboestrol on day 2.

Table 2. *Yields of sodium stilboestrol monoglucuronide from rabbit urine*

(Amount of stilboestrol fed was 4·8 g. (6 rabbits). Total Na salt recovered was 5·37 g.)

	Sodium salt from	
Day	Centrifuged urine (g.)	Urinary deposit (g.)
1	0·48	0·65
2	0·70	1·88
3	0·40	0·61
4	0·25	0·40
Totals	1·83	3·54

Benzylamine salt of stilboestrol glucuronide. Pure stilboestrol glucuronide (200 mg.) was dissolved in 20 ml. ethanol followed by 20 ml. ethyl acetate. Benzylamine (10 drops) was then added to the mixture with stirring. After a few seconds the *salt* crystallized out. After 15 min. at 0°, the mixture was filtered, and the salt washed with ethanol and dried in the desiccator (yield 210 mg., m.p. 218° decomp.). On recrystallization from 10% ethanol, the salt formed long white needles, m.p. 223° (decomp.), and showed $[\alpha]_D^{20^\circ} = -55{\cdot}3^\circ$ ($c=0{\cdot}172$ in 50% aqueous acetone). (Found: C, 63·75; H, 6·9; N, 2·55%. $C_{31}H_{37}O_8N.2H_2O$ requires C, 63·4; H, 7·0; N, 2·4%.) The salt was sparingly soluble in all the usual solvents.

Detection of free stilboestrol. (*a*) *In urine.* Urine from rabbits receiving stilboestrol orally was centrifuged and the deposit removed. It was then acidified and extracted with ether in a continuous extractor for several hours. The glucuronide in the ether extract was removed by six treatments with saturated sodium bicarbonate solution. The ether was now extracted with 10% (w/v) Na_2CO_3 to remove free stilboestrol. Acidification of this latter extract, followed by extraction with ether, yielded a solution giving a red colour in the Dingemanse reaction thus indicating the presence of free stilboestrol. A separate experiment showed that stilboestrol glucuronide could be completely removed, as indicated by the Dingemanse reaction, from an ethereal solution by saturated $NaHCO_3$. By this method small amounts of free stilboestrol were found in the urine for 4 days after dosing.

(*b*) *In faeces.* By a similar method free stilboestrol in small amounts was detected in the faeces of rabbits on all 4 days after dosing.

Experiments with hexoestrol

Quantitative results. These are given in Table 3, and Fig. 2 illustrates a typical experiment. These figures indicate that about 71% of hexoestrol is excreted as a glucuronide, the excretion persisting for 4–5 days after dosing. The ethereal sulphate and neutral sulphur outputs are not raised.

Table 3. *The excretion of glucuronic acid, ethereal sulphate and neutral sulphur by rabbits receiving hexoestrol orally*

Rabbit no.	Dose (mg./kg.)	Extra glucuronic acid (% of dose)	Extra ethereal sulphate (% of dose)	Extra neutral S (% of dose)
85	200	77·1	—	—
87	200	73·1	—	—
97	200	63·4	—	—
89	250	—	0	0
90	250	—	0	0

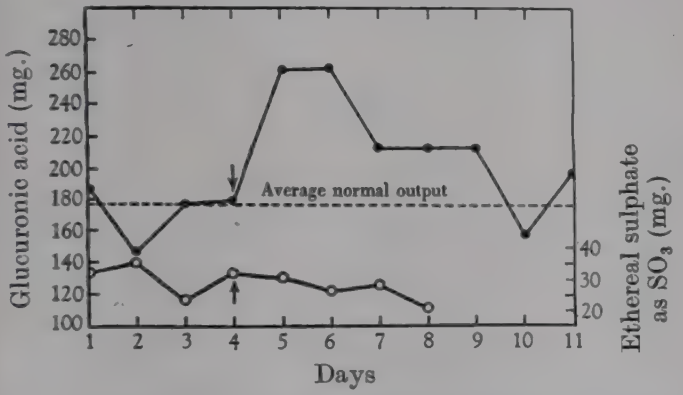

Fig. 2. The glucuronic acid and ethereal sulphate output in rabbits receiving hexoestrol orally. ●—●, glucuronic acid curve for rabbit no. 85 that received 558 mg. of hexoestrol on day 4. ○—○, ethereal sulphate curve for rabbit no. 90 that received 770 mg. of hexoestrol on day 4.

Isolation of p-*β*-D-*glucuronosidodiphenyl.* Preliminary experiments on the isolation of hexoestrol glucuronide indicated that its properties were not similar to those of stilboestrol glucuronide. In order to avoid undue wastage of hexoestrol, model experiments were first carried out on *p*-hydroxydiphenyl since it was thought that its glucuronide might be similar to that of saturated hexoestrol.

p-Hydroxydiphenyl (1 g.) (m.p. 164–165°; British Drug Houses Ltd.) suspended in water was fed to a rabbit by stomach tube. The urine (150 ml.) was collected after 24 hr. It gave a strong naphthoresorcinol reaction. On acidifying with HCl a fine white precipitate separated which could not be easily filtered. Extraction of the urine with ether yielded no glucuronide. The urine was therefore extracted three times in a separating funnel with a 1 : 3 ethanol-ether mixture. The extract was evaporated and a crystalline residue (0·75 g.) was left. In a similar manner, the urine for the second day yielded 155 mg. of the material. The whole was recrystallized from a large volume of hot water and p-*β*-D-*glucuronosidodiphenyl* (*p*-phenylphenylglucuronide) crystallized as needles, m.p. 189°, showing $[\alpha]_D^{20^\circ} = -90{\cdot}6^\circ$ ($c=1$ in 0·1 N-NaOH). (Found: C, 58·1; H, 5·6%. $C_{18}H_{18}O_7.1\frac{1}{2}H_2O$ requires C, 57·9; H, 5·7%.) It gives a strong naphthoresorcinol reaction, but only after heating with HCl and naphthoresorcinol for 2 min., i.e. twice the usual heating time (cf. dienoestrol glucuronide).

Benzylamine salt of p-*glucuronosidodiphenyl.* Ten drops of benzylamine were added gradually with stirring to 100 mg. of glucuronosidodiphenyl dissolved in 30 ml. of an equal mixture of ethanol and ethyl acetate. After 2 hr. at 0° the separated *benzylamine salt* was filtered and washed with a little ethanol (yield, 100 mg.). On crystallization from dilute aqueous ethanol, it formed colourless needles, m.p. 205° (decomp.) and showed $[\alpha]_D^{20^\circ} = -69{\cdot}7^\circ$ ($c=1$ in 0·1 N-NaOH). (Found: C, 65·5; H, 6·15; N, 2·9%. $C_{25}H_{27}O_7N.\frac{1}{2}H_2O$ requires C, 64·9; H, 6·2; N, 3·0%.) This salt is more soluble in cold water than the corresponding salts of the oestrogens.

Hexoestrol monoglucuronide. Hexoestrol (1 g.) (*meso* - 4 : 4′ - dihydroxy - *γδ* - diphenyl hexane, m.p. 184°; British Drug Houses Ltd.) was fed by stomach tube to each of five rabbits. The urine was collected daily for 4 days, each day's urine being worked up separately. Extraction with ether yielded very little material and the urine gave a strong naphthoresorcinol reaction only after prolonged boiling. Each day's urine was worked up as follows.

The urine (600–800 ml.) was acidified with 10 N-H_2SO_4 and then shaken with 200 ml. of a 1 : 5 ethanol-ether mixture. A copious white precipitate of inorganic material separated and this was filtered off at the pump. The filtrate was saturated with $(NH_4)_2SO_4$ thus causing an effective separation of the ethanol-ether layer which was removed. The urine layer was extracted with two further 200 ml. portions of ethanol-ether. The combined extracts (dried by anhydrous Na_2SO_4) were taken to dryness *in vacuo* at 60°. A clear brown oil remained (which crystallized in some experiments). This was boiled with 30 ml. water and, after adding charcoal, filtered. On cooling, the glucuronide separated as an oil. The oil was redissolved in the water which was then saturated with $(NH_4)_2SO_4$ and the glucuronide extracted with 1 : 5 ethanol-ether. Removal of the solvents left a partially crystalline mass which further crystallized on triturating with ether. The crystals were filtered off and the mother liquor evaporated to a gum which was used for making the benzylamine salt.

The total yield of crystalline material was 0·5 g. On recrystallization from water or benzene-acetone, *hexoestrol mono-β-D-glucuronide trihydrate* was obtained as colourless needles, m.p. 183–184° (decomp.) after sintering at 100° and showing $[\alpha]_D^{20^\circ} - 66{\cdot}3^\circ$* ($c=2$ in 0·1 N-NaOH). (Found: C, 57·8;

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* This rotation is quoted tentatively, since we suspect resolution during recrystallization (see discussion).

H, 6·95 %; glucuronic acid, 39·3 %. $C_{24}H_{30}O_8 . 3H_2O$ requires C, 57·6; H, 7·25 %; glucuronic acid, 38·8 %.) The pure compound was insoluble in ether, benzene, light petroleum and chloroform and sparingly soluble (< 1 %) in ethanol, acetone and cold water. It gives a strong naphthoresorcinol test on prolonged boiling. Since it is insoluble in chloroform, the Dingemanse $SbCl_5$ test cannot be carried out in the ordinary way. On suspending the glucuronide in dry chloroform and then warming the solution with an excess of 25 % $SbCl_5$ in chloroform a pale carmine colour is produced similar to that obtained with hexoestrol itself.

The benzylamine salt of hexoestrol monoglucuronide. Some of the residual gum obtained above after separation of crystalline hexoestrol glucuronide was dissolved in a mixture of ethanol and ethyl acetate (1 : 1). A slight excess of benzylamine was added drop by drop with stirring. No crystals separated. The mixture was therefore warmed on a water bath and on cooling and stirring, a crystalline solid separated. The crystals (m.p. 218°) were filtered and washed with absolute ethanol and dried in a desiccator. The *salt* on recrystallization from dilute aqueous ethanol formed colourless needles, m.p. 222°, and showed $[\alpha]_D^{20^\circ} = -48{\cdot}7^\circ$ ($c = 0{\cdot}5$ in 2N-HCl in 50 % aqueous ethanol). (Found: C, 62·8; H, 7·0; N, 2·3 %. $C_{31}H_{30}O_8N . 2H_2O$ requires C, 63·1; H, 7·35; N, 2·4 %.) It gave a strong naphthoresorcinol reaction on prolonged boiling, and a pale carmine colour in the Dingemanse reaction carried out as for hexoestrol glucuronide. It was insoluble in ethanol, ethyl acetate and cold water, but soluble in hot water.

Table 4. *The glucuronic acid output in rabbits receiving dienoestrol orally*

		Glucuronic acid output		
Rabbit no.	Dose (mg./kg.)	Average normal (mg./day)	Average output/day for 3 days after dosing (mg./day)	Increase (mg.)
81	200	127	132	15
82	200	154	150	—
83	200	135	146	33
84	200	160	224	192

Table 5. *Ethereal sulphate and neutral sulphur output in rabbits receiving dienoestrol orally*

Rabbit no.	Dose (mg./kg.)	Ethereal sulphate increase	Neutral S increase
98	250	0	—
68	250	0	—
89	250	0	0
90	250	0	0
87	250	0	0
81	250	0	0

Experiments with dienoestrol

Quantitative results. These are given in Tables 4 and 5 and single experiments are illustrated in Fig. 3. These tables show that dienoestrol does not disturb the ethereal sulphate and neutral sulphur output. In the case of glucuronic acid, Table 4 shows that in three cases there is apparently no disturbance of the normal glucuronic acid output. In the fourth case there is an appreciable rise corresponding to nearly 50 % of the dose. Qualitative experiments (see below) have shown that a glucuronide is actually excreted and the negative results in most of our quantitative glucuronic acid estimations can be ascribed to the stability of the glucuronide to acid hydrolysis. Stable glucuronides which cannot be quantitatively estimated by the naphthoresorcinol method have been described on previous occasions, e.g. euxanthic acid, *o*-aminophenylglucuronide and *p*-sulphonamido-*o*-aminophenylglucuronide (Hanson *et al.* 1944). The behaviour of dienoestrol monoglucuronide in the naphthoresorcinol procedure is being further investigated.

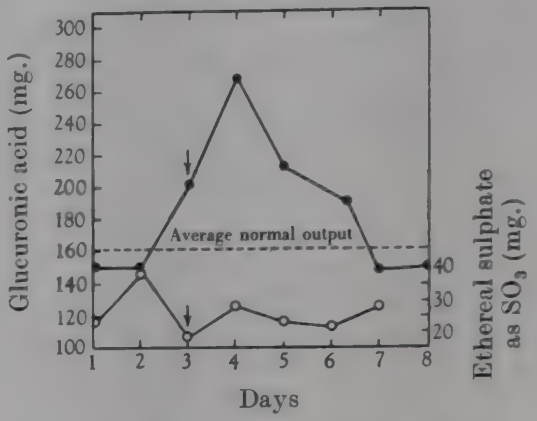


Fig. 3. The glucuronic acid and ethereal sulphate output in rabbits receiving dienoestrol orally. ●—●, glucuronic acid curve for rabbit no. 84 that received 556 mg. dienoestrol on day 3. ○—○, ethereal sulphate curve for rabbit no. 87 that received 930 mg. of dienoestrol on day 3.

Dienoestrol monoglucuronide. Preliminary experiments showed that a single oral dose of 1 g. of dienoestrol (m.p. 238–239°) was not readily absorbed by rabbits and much of the compound appeared unchanged in the faeces. Therefore six rabbits were each given four separate doses of 0·25 g. during 2 days (at 11 a.m. and 4 p.m. each day). The urines were worked up daily for 5 days after the first dose, by the same method as was used for hexoestrol glucuronide. From the ether-ethanol extracts dienoestrol glucuronide was obtained as an oil which partially crystallized on triturating with ether. The residual urines after ether-ethanol extractions were then subjected to prolonged extraction (30 hr.) with ether in continuous extractors. In this way a total of 3·5 g. of crystalline glucuronide was obtained and 0·32 g. of the benzylamine salt was prepared from residues (cf. hexoestrol glucuronide). On repeated recrystallization from acidulated water *dienoestrol mono-β-D-glucuronide monohydrate* separated as colourless needles, m.p. 181–183° (decomp.) after sintering at 120–135° and showing $[\alpha]_D^{20°}$ $-45{\cdot}9°$ ($c=1$ in 0·1N-NaOH). (Found: C, 62·65; H, 6·15%. $C_{24}H_{26}O_8.H_2O$ requires C, 62·6; H, 6·1%.)

The glucuronide is insoluble in ether, benzene and light petroleum, sparingly soluble in cold water, acetone and ethanol, but fairly soluble in the latter solvents when hot. A suspension of the glucuronide in chloroform gives the same magenta colour with $SbCl_5$ (Dingemanse reaction) as does the parent oestrogen. It is remarkably stable to acid hydrolysis for it can be recrystallized from acidulated water without hydrolysis. In the qualitative naphthoresorcinol test in which HCl of at least 5N strength is used, heating for 5 min. is necessary to get a positive reaction. If the naphthoresorcinol test is carried out on the glucuronide in the normal manner, i.e. 1 min. heating, the test is negative.

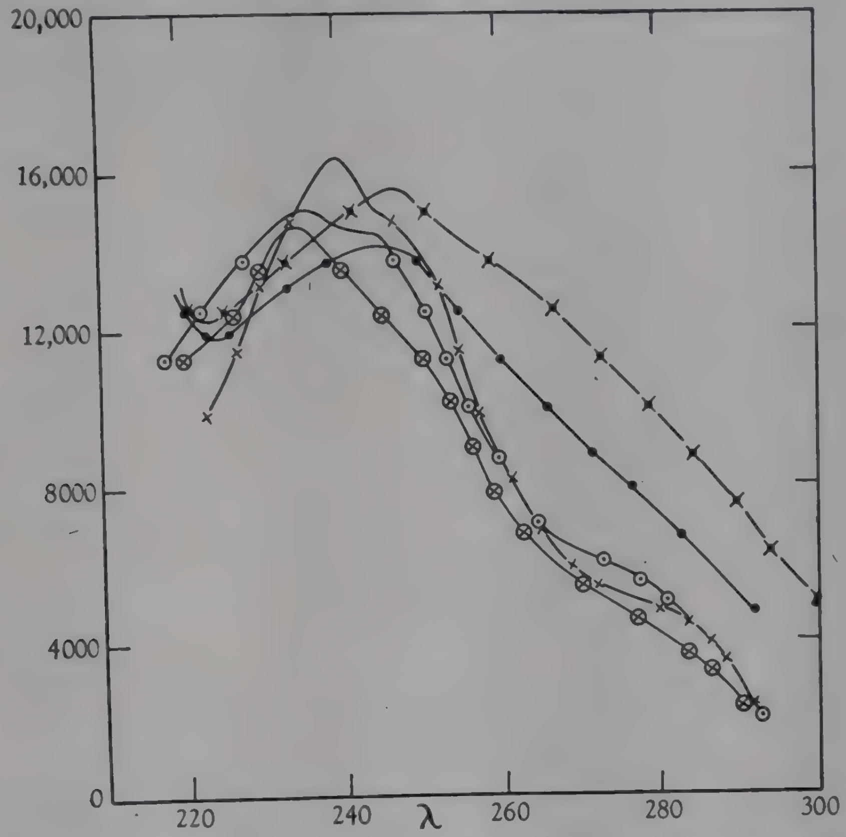


Fig. 4. Ultraviolet absorption spectra of stilboestrol and stilboestrol glucuronide. ×—× stilboestrol in absolute ethanol. ⊙—⊙ stilboestrol in 50% (v/v) aqueous ethanol + 1 equiv. KOH. •—• stilboestrol in 50% (v/v) aqueous ethanol + 5 equiv. KOH. ⋊—⋊ stilboestrol in 50% (v/v) aqueous ethanol + excess 0·005M-KOH. ⊗—⊗ stilboestrol glucuronide in absolute ethanol.

The benzylamine salt of dienoestrol glucuronide. This *salt* was prepared from the gummy residues after crystallization of the free glucuronide as described for hexoestrol. It was recrystallized from dilute aqueous ethanol and formed colourless needles, m.p. 223° (decomp.), and showed $[\alpha]_D^{20°} = -35{\cdot}4$ ($c=0{\cdot}5$ in 0·1N-NaOH). (Found: C, 63·2; H, 6·7; N, 2·6%. $C_{31}H_{35}O_8N.2H_2O$ requires C, 63·6; H, 6·7; N, 2·4%.) It behaves in the same way as the parent glucuronide in the Dingemanse and naphthoresorcinol reactions.

Spectroscopic observations

Stilboestrol. A number of workers have studied the ultraviolet absorption spectrum of stilboestrol. Elvidge (1939) reported a single maximum in the absorption curve at 240 mμ. with $\epsilon_{max.}$ 15,500–15,800. In a later paper (Elvidge, 1940) $\lambda_{max.}$ is given as 239 mμ. with $\epsilon_{max.}$ 16,000. Kharasch & Kleiman (1943) reported $\lambda_{max.}$ 250 mμ. Solmssen (1943) reported a maximum at 240 mμ. with $\epsilon_{max.}$ 15,700 and an inflexion near 280 mμ., $\epsilon = 7000$. The occurrence of this inflexion was confirmed by Kaiser & Koenig (1946), but they recorded the maximum at 250 mμ.

The sample of stilboestrol used in the present work showed in ethanol solution $\lambda_{max.}$ 240 mμ., $\epsilon_{max.}$ 16,400, with inflexions near 245 mμ., $\epsilon = 15,000$; and 270–280 mμ., $\epsilon = 5000$ (Fig. 4).

The monoglucuronide shows $\lambda_{max.}$ 235 mμ., $\epsilon_{max.}$ 14,600 with an inflexion 270–280 mμ., $\epsilon = 5000$ (Fig. 4).

The ultraviolet absorption spectrum has also been studied in aqueous ethanol containing various amounts of potassium hydroxide, in an attempt to obtain information regarding the ionization of the phenolic hydroxyl groups which might be correlated with the ease of biological conjugation (Fig. 4).

In 50% (v/v) aqueous ethanol containing one equivalent of KOH, the main absorption band of stilboestrol splits into two bands: $\lambda_{\text{max.}}$ 235 mμ., $\epsilon_{\text{max.}}$ 15,000; $\lambda_{\text{max.}}$ 244 mμ., $\epsilon_{\text{max.}}$ 14,500. The inflexion near 280 mμ. remains unchanged. Further addition of alkali results in the longer wave length component becoming predominant together with the disappearance of the 280 mμ. inflexion. This variation in the position of $\lambda_{\text{max.}}$ with solvent between 235 and 250 mμ. may account for the lack of agreement between different workers regarding the actual position of the band. Furthermore, if the shift towards longer wave lengths with increasing pH of the solvent is ascribed to increased ionization of the stilboestrol, then the presence of an inflexion near 245 mμ. in ethanol solution and in a solution buffered at pH 7·4 is indicative of some ionization under these conditions.

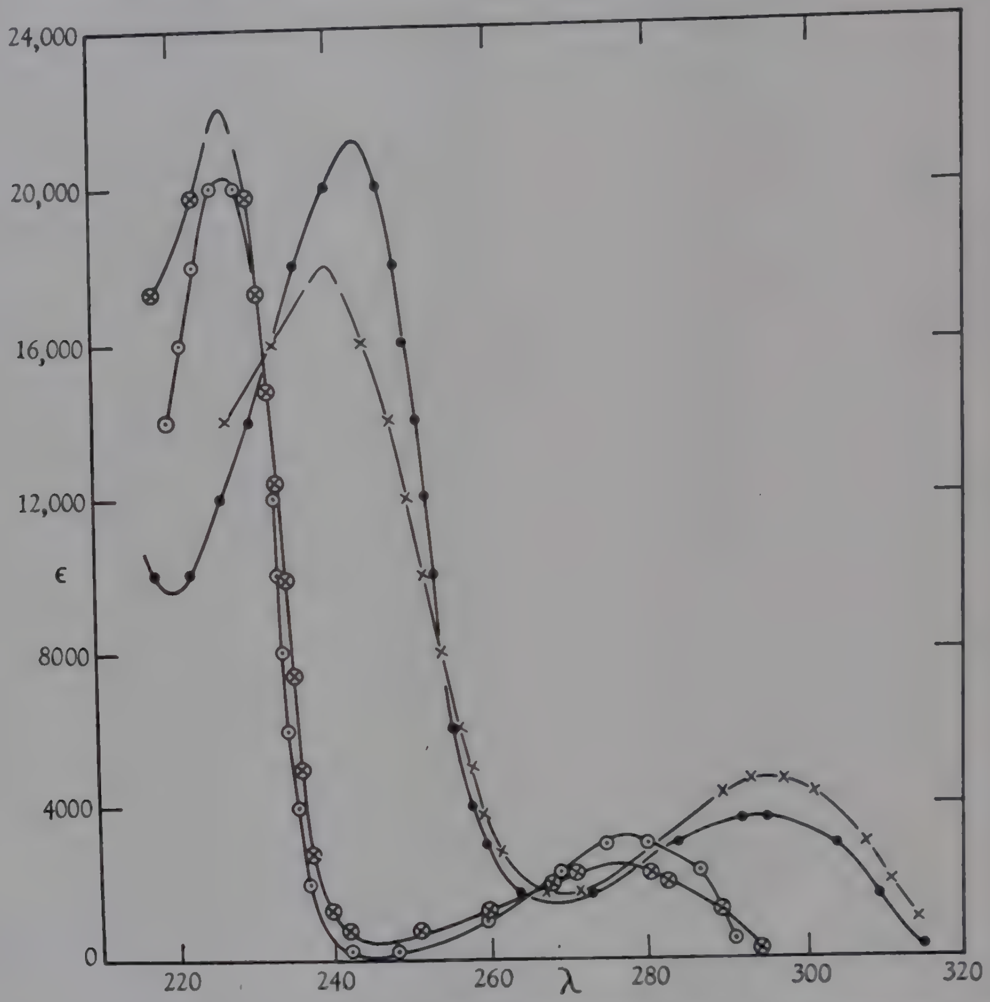


Fig. 5. Ultraviolet absorption spectra of hexoestrol and hexoestrol glucuronide. ×—× hexoestrol in 50% (v/v) aqueous ethanol. ⊙—⊙ hexoestrol in 50% (v/v) aqueous ethanol + 1 equiv. KOH. ●—● hexoestrol in 50% (v/v) aqueous ethanol + excess 0·005 M-KOH. ⊗—⊗ hexoestrol glucuronide in absolute ethanol.

Hexoestrol. Elvidge (1939) reported two maxima in the absorption spectrum of hexoestrol $\lambda\lambda_{\text{max.}}$ 229 and 281 mμ. Kaiser & Koenig (1946) recorded $\lambda\lambda_{\text{max.}}$ 230 and 280 mμ.

It has been found, however, that the actual position of $\lambda_{\text{max.}}$ varies with the solvent. In 50% (v/v) aqueous ethanol the maxima occur at 240 and 295 mμ. with $\epsilon_{\text{max.}}$ 18,000 and 4700 respectively (Fig. 5). Addition of one equivalent of KOH to this solution shifts the spectrum to shorter wave lengths, $\lambda\lambda_{\text{max.}}$ 227·5 and 278 mμ. with $\epsilon_{\text{max.}}$ 20,200 and 3200 respectively (Fig. 5). Further addition of alkali up to five equivalents has little effect, but in excess of alkali (0·005 M in a solution $\text{M} \times 10^{-4}$ with respect to hexoestrol) the spectrum reverts to that of hexoestrol in aqueous ethanol, showing $\lambda\lambda_{\text{max.}}$ 243·5 and 293·5 mμ. with $\epsilon_{\text{max.}}$ 21,200 and 3700 respectively (Fig. 5).

Hexoestrol glucuronide in ethanol shows $\lambda\lambda_{\text{max.}}$ 227 and 276 mμ. with $\epsilon_{\text{max.}}$ 22,000 and 2400 respectively (Fig. 5).

It is difficult to interpret these results on the basis of ionization of hexoestrol, particularly since the shift to shorter wave lengths on addition of one equivalent of KOH is contrary to the usual behaviour of phenolic compounds. (Phenol itself shows $\lambda\lambda_{max.}$ 215 and 273 mμ. in ethanol solution and $\lambda\lambda_{max.}$ 230 and 285 mμ. in alkali.) It seems more likely that the variations are to be ascribed to a solvent effect only, the effects of any ionization changes being subsidiary.

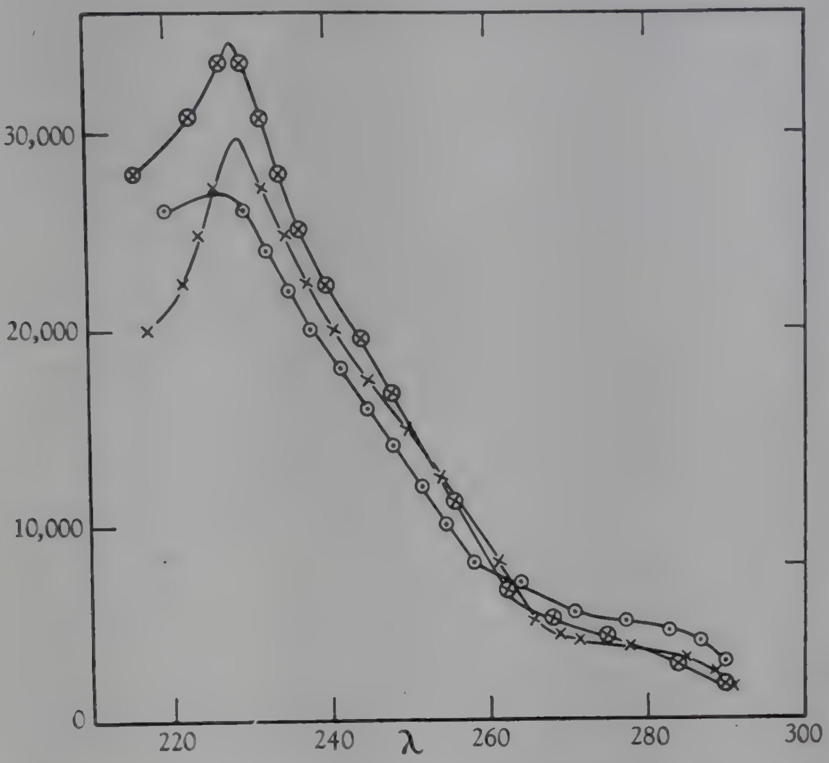


Fig. 6. Ultraviolet absorption spectra of dienoestrol and dienoestrol glucuronide. × — × dienoestrol in absolute ethanol. ⊙—⊙ dienoestrol in 50% (v/v) aqueous ethanol + 1 equiv. KOH. ⊗—⊗ dienoestrol glucuronide in absolute ethanol.

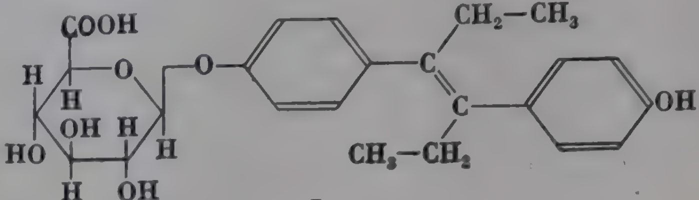
Dienoestrol. The sample of dienoestrol shows an absorption spectrum similar in general shape to that of stilboestrol, with $\lambda_{max.}$ 229 mμ., $\epsilon_{max.}$ 30,000. There is also an inflexion in the curve between 275 and 280 mμ. with $\epsilon = 4000$ (Fig. 6). Elvidge (1940) gives $\lambda_{max.}$ 228 mμ., $\epsilon_{max.}$ 26,600. There is little change in spectrum with variation in solvent pH, whilst the glucuronide shows $\lambda_{max.}$ 228 mμ., $\epsilon_{max.}$ 34,700, with an inflexion near 275 mμ., $\epsilon = 4000$ (Fig. 6).

These spectra give little information regarding any possible ionization of the dienoestrol molecule. It is probable that the absorption maximum at 229 mμ. has its origin in the ethylenic double bonds, and is little affected by ionization of the phenolic hydroxyls.

DISCUSSION

The present results indicate that in the rabbit the main metabolic change undergone by the three synthetic oestrogens studied is conjugation with glucuronic acid to form monoglucuronides. In the cases of stilboestrol and hexoestrol quantitative measurements show that at least 70% of the drugs is conjugated with glucuronic acid and this is probably true also for dienoestrol. This conjugation probably results in a detoxication and an inactivation of these compounds. Not one of the three compounds appears, from quantitative data, to form a mercapturic acid or an ethereal sulphate. The absence of mercapturic acid formation is not surprising for all three are para-substituted compounds and therefore the position in the aromatic ring where acetylcysteine conjugation normally occurs is blocked. Furthermore there is no known case of an aromatic hydroxy compound forming a mercapturic acid. However, the apparent absence of ethereal sulphate conjugation is surprising, for all three compounds contain phenolic hydroxyl groups, the prerequisites for sulphate conjugation. There is of course the possibility that ethereal sulphates are slowly formed and excreted, but that their concentration in the urine is too low to be detected by the quantitative method used. This possibility is being investigated. If the absence of ethereal sulphates is confirmed, then we must look to the nature of the hydroxyl groups for the reason, for phenols are known which, when fed to animals, do not conjugate as ethereal sulphate, e.g. salicylic acid (Williams, 1938). In the case of salicylic acid the phenolic hydroxyl is not free as a result of hydrogen bonding. It is possible that ethereal sulphate formation is related to the ionization of phenolic hydroxyl groups for it takes place only with phenols. Ethereal sulphate formation with other types of hydroxyl groups, such as those found in alcohols, has never been recorded. In the alcohols it is known that the OH groups are extremely feebly ionized (e.g. glycol, $K_a = 6 \times 10^{-15}$; glycerol, $K_a = 7 \times 10^{-15}$) whereas in phenols the ionization constants vary between $K_a = 5{\cdot}6 \times 10^{-8}$ for p-nitrophenol to about 1×10^{-10} for phenol (see Britton, 1942). This suggests a small optimum ionization of the OH group for ethereal sulphate formation. The ionization constants of the synthetic oestrogens have not been recorded. Our spectroscopic data indicate that stilboestrol will ionize in alkaline solution, but the data for hexoestrol and dienoestrol do not permit of any conclusion in this respect. The relation between ionization constants and ethereal sulphate formation is being further investigated.

That stilboestrol has a *trans*-structure has, however, been questioned on spectroscopic grounds by Solmssen (1943).



I

In the case of hexoestrol, the position is not quite so clear, since hexoestrol is an internally compensated compound, i.e. *meso*-3:4-di(p-hydroxyphenyl)-hexane. Conjugation of one of the hydroxyls of hexoestrol with glucuronic acid destroys this internal compensation and therefore two enantiomorphous hexoestrol monoglucuronides (II and III) should arise. This point is being further investigated, for it is quite possible that the glucuronide isolated is a racemate.

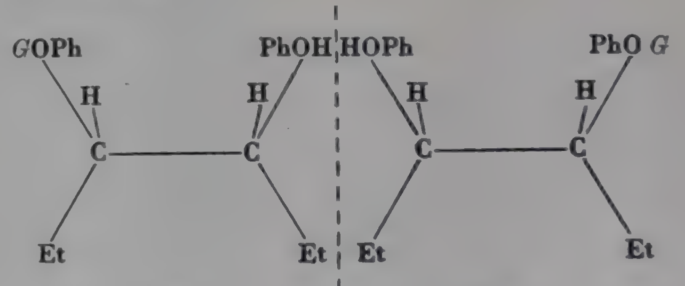
Table 6. *The properties of the monoglucuronides of synthetic oestrogens*

Property	Stilboestrol glucuronide		Hexoestrol glucuronide		Dienoestrol glucuronide	
Hydration	$2H_2O$		$3H_2O$		$1H_2O$	
M.p.	179°		183–184°		181–183°	
$[\alpha]_D$	−56·5° (in ethanol)		−66·3°* (in 0·1 N-NaOH)		−45·9° (in 0·1 N-NaOH)	
Ultraviolet absorption spectra in ethanol	$\lambda_{\text{max.}}$	$\epsilon_{\text{max.}}$	$\lambda_{\text{max.}}$	$\epsilon_{\text{max.}}$	$\lambda_{\text{max.}}$	$\epsilon_{\text{max.}}$
	235 mμ.	14,600	227 mμ.	22,000	228 mμ.	34,700
	~270–280 mμ.	5,000	276 mμ.	2,400	~275 mμ.	4,000
Stability to acid hydrolysis	Easily hydrolyzed		Hydrolyzed with some difficulty		Hydrolyzed with much difficulty	
M.p. of benzylamine salt	223°		222°		223°	

* See discussion.

The nature of the glucuronides of the synthetic oestrogens also calls for some comment (see Table 6). They vary in their stability to acid hydrolysis, for whereas stilboestrol glucuronide is relatively easily hydrolyzed, dienoestrol glucuronide is hydrolyzed only with difficulty, hexoestrol glucuronide taking an intermediate position. All three are hydrated which is a common feature of many glucuronides. It has been frequently observed in this laboratory that a large number of glucuronides do not crystallize without water of crystallization and removal of this water usually results in the production of a non-crystalline glass. The negative rotations of the three glucuronides indicate that, like all biosynthetic glucuronides, they are β-glycosides.

The exact structures of these glucuronides have yet to be determined for a consideration of the stereochemistry of hexoestrol and dienoestrol indicates that the biosynthesis of more than one monoglucuronide is possible in these cases. The structure of stilboestrol glucuronide is straightforward because, if we accept a *trans*-stilbene structure for stilboestrol, then the glucuronide can be formulated as 4-(β'-D-glucuronosido-)-4'-hydroxy-α:β-diethyl-*trans*-stilbene (I).



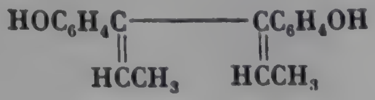
II III

where —PhOG = —$C_6H_4OC_6H_9O_6$.

Similar difficulties arise with dienoestrol, but for slightly different reasons. As far as we are aware, the configuration of dienoestrol has not been established, but we know that it is a 3:4-di(p-hydroxyphenyl)-hexa-2:4-diene.

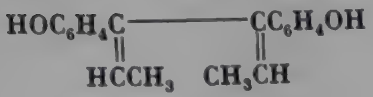
If it has structure (IV), then two monoglucuronides are possible depending upon which hydroxyl is conjugated. With structures (V) and (VI) only one monoglucuronide is possible in each case. These points are being further investigated.

Stroud (1939*b*) has shown that stilbene when injected into rabbits gives rise to hippuric acid, which indicated splitting and oxidation at the double bond.



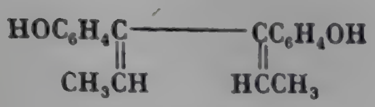
$$\underset{\overset{\|}{HCCH_3}}{HOC_6H_4C}-\!\!-\!\!-\underset{\overset{\|}{HCCH_3}}{CC_6H_4OH}$$

IV



$$\underset{\overset{\|}{HCCH_3}}{HOC_6H_4C}-\!\!-\!\!-\underset{\overset{\|}{CH_3CH}}{CC_6H_4OH}$$

V



$$\underset{\overset{\|}{CH_3CH}}{HOC_6H_4C}-\!\!-\!\!-\underset{\overset{\|}{HCCH_3}}{CC_6H_4OH}$$

VI

A similar change is therefore possible with stilboestrol, the product in this case being *p*-hydroxybenzoic acid. We have examined stilboestrol urine for *p*-hydroxybenzoic acid, but were unable to find it. We conclude therefore that, in the rabbit, the double bond in stilboestrol is resistant to biological attack.

SUMMARY

1. A preliminary study has been made of the fate of stilboestrol, hexoestrol and dienoestrol in the rabbit.

2. The preparation and properties of the monoglucuronides of stilboestrol, hexoestrol and dienoestrol prepared by feeding these oestrogens to rabbits are described.

3. The benzylamine salts of the above glucuronides have been prepared and are described.

4. The isolation of *p*-glucuronosidodiphenyl from the urine of a rabbit receiving *p*-hydroxydiphenyl is described, together with its benzylamine salt.

5. Some 70 % of stilboestrol and of hexoestrol is excreted by rabbits combined with glucuronic acid. In the case of dienoestrol the amount conjugated is uncertain because dienoestrol monoglucuronide is highly resistant to acid hydrolysis and hence its determination by the method of Hanson, Mills & Williams (1944) is uncertain.

6. None of the three oestrogens affects the ethereal sulphate and neutral sulphur level of rabbit urine. These results are discussed and the possible reasons for them indicated.

7. The absorption spectra of the three oestrogens and their glucuronides under various conditions have been studied.

8. There is no indication that stilboestrol is degraded *in vivo* to single ring compounds.

9. The stereochemistry of hexoestrol and dienoestrol glucuronides is discussed.

The work was carried out at the request of the Oestrogens in Cancer Committee (Chemical Panel) of the Royal Society of Medicine. The synthetic oestrogens used in this work were given by the British Empire Cancer Campaign. One of us (A.L.S.) participated in this work as a holder of an I.C.I. Fellowship.

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1948

The Estimation of a Fraction Resembling Pregnanediol Glucuronide in Human Urine

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The amount of pregnanediol in human urine can be estimated by an assay of the (partially purified) sodium pregnanediol glucuronidate (Na*PG*) extracted by butanol (Venning, 1937, 1938), or of the pregnanediol itself, extracted from the urine after hydrolytic treatment (Astwood & Jones, 1941; Talbot, Berman, MacLachlan & Wolfe, 1941). The latter method is capable of considerable specificity and sensitivity and has been extensively developed. Jayle, Crépy & Wolf (1943) and Jayle & Libert (1946) used methods in which the glucuronidate, purified after isolation from butanol extracts, was estimated by the naphthoresorcinol reaction, and Allen & Viergiver (1941) precipitated the glucuronidate from butanol extracts with lead nitrate and estimated reducing substances after acid hydrolysis.

The present work arose out of an attempt to improve existing methods, and it was decided in the first place to employ the naphthoresorcinol reaction for estimating glucuronide. According to Bucher & Geschickter (1941), free pregnanediol apparently does not occur in human urine. It seemed to us that the method of Jayle *et al.* (1943) incorporated many of the known sources of error of the original Venning method, which is admittedly not suitable for very small amounts of Na*PG*, whilst that of Allen & Viergiver (1941) likewise seemed better adapted for assay of quantities larger than those which we wished to investigate. We have, therefore, devised a technique in which the glucuronidate is 'entrained' (presumably as the barium salt, cf. Westphal, 1944) on a precipitate of barium phosphate, and is estimated after isolation in this way. This method enabled us to obtain accurate recoveries of small amounts of Na*PG* after butanol extraction from pure solutions. It was found, however, that when applied to urine of normal men, or of women in the follicular phase of the menstrual cycle, it indicated the presence of glucuronidate, resembling Na*PG* closely in its solubility in butanol and its behaviour on entrainment, in great excess of the amount of Na*PG* which would be expected. The total glucuronide found in this fraction we have called 'pregnanediol glucuronide-like glucuronide', or *pregnanediol-like glucuronide* (*PLG*) (Bisset, Brooksbank & Haslewood, 1947) and we have attempted to estimate it. We have introduced a modification into the naphthoresorcinol method.

METHODS

(Including results of standardization of the method of estimating Na*PG*)

Abbreviations. Na*PG*(V) = a crystalline specimen of glucuronidate, prepared by the method of Venning (1937, 1938). Na*PG*(M) = purified glucuronidate, freed from ketonic material (Sutherland & Marrian, 1947). We found 1 mg. of Na*PG*(V) ≡ 0·859 mg. Na*PG*(M) ≡ 0·777 mg. of anhydrous sodium pregnanediol glucuronidate (Na*PG*) (Marrian & Gough, 1946). 1 mg. Na*PG* ≡ 0·618 mg. of pregnanediol. Na*PG*(V) was kept in a vacuum desiccator over H_2SO_4 until weighed out for use. Analysis indicated that, after this treatment, Na*PG*(V) contained about 1·5 mol. H_2O. Solutions of sodium pregnanediol glucuronidate were kept at 0–5°.

Purification of butanol. n-Butanol (Commercial Solvents Ltd.) was distilled at 112–118° over KOH (5 g./l.). It was then redistilled at *c.* 70°/20 mm., with an air leak and in the presence of 1 g./l. of 4-aminophenol and 1 ml./l. of 5 N-NaOH. It was finally redistilled at *c.* 60–70°/20 mm. This process removed substances sometimes present which tended to destroy Na*PG* when solutions with butanol were evaporated *in vacuo*.

Test of purified butanol. A volume of 250 ml., with 2 or 3 ml. of Na*PG*(V) solution (50 mg./100 ml.), was completely evaporated *in vacuo* as described below for urine extracts. The residue was heated to *c.* 90° with 10 ml. of water, the cooled solution was filtered and the glucuronidate in 2 ml. of the filtrate (≡ 0·2 mg. or 0·3 mg. Na*PG*(V)) was entrained and estimated as described under 'entrainment of glucuronidate' (below). Allowing for known losses, a recovery of not less than 95% was considered satisfactory.

Naphthoresorcinol solution. A small excess over the amount of naphthoresorcinol (Hopkin and Williams) needed for the day's experiments was weighed out and dissolved at *c.* 50° in water to make a 0·025 M-solution, which was then filtered. In earlier experiments, naphthoresorcinol solution (0·025 M) was made and 'aged' as described, e.g., by Maughan, Evelyn & Browne (1938) and Hanson, Mills & Williams (1944): this procedure is discussed below.

The naphthoresorcinol reaction

It is clear from the literature that there is little agreement as to the best method of preparing the naphthoresorcinol reagent. We have found that the amount of final extractable colour produced with a given quantity of Na*PG* varies with the age of the naphthoresorcinol solution used, reaching a peak after about 2 days' storage at 0–5°, and then tending to become less: the reagent 'blank' increases (cf. Maughan *et al.* 1938). With old naphthoresorcinol solutions the final colour tends to be obviously more red. We have found also that fresh naphthoresorcinol solution may give very inconstant amounts of colour with the same amount of Na*PG*. This state of affairs militates seriously against the accuracy and reproducibility of the method. The facts suggest that the actual formation of coloured matter in the reaction is not in fact due to interaction of naphthoresorcinol and glucuronic acid, but rather that the effective reagent is some unstable oxidation product of naphthoresorcinol; this product, we found, is not, as appeared possible, 3-hydroxy-1:4-naphthoquinone. We therefore considered the possibility of adding to fresh naphthoresorcinol solution a weak oxidizing agent and thus effecting at once a reproducible 'ageing' process. Dr R. V. G. Ewens suggested, *inter alia*, $K_3Fe(CN)_6$ for this purpose and we found it satisfactory. One ml. of 0·05M-$K_3Fe(CN)_6 \equiv$ 2 ml. of naphthoresorcinol solution (0·025M). We therefore first tried the effect of adding 0·1, 0·2, 0·4. . . 1·0 ml. of 0·05M-$K_3Fe(CN)_6$ to the reaction tubes. With 0·6–1·0 ml. $K_3Fe(CN)_6$ the final colours were increasingly red and the reagent 'blank' much increased. The use of 0·1 ml. 0·05M-$K_3Fe(CN)_6$, however, gave an appearance during the reaction very similar to that given by 'aged' naphthoresorcinol solutions, a low 'blank' and constancy of colour. We therefore used this amount, and employed fresh, filtered naphthoresorcinol solution, as described above.

Estimation of Na*PG*. Quantities were measured into wide test tubes (15 × 2 cm.) as follows:

Test	Blank
Na*PG* solution + water to make 1·4 ml.	1·4 ml. water
0·1 ml. 0·05M-$K_3Fe(CN)_6$	0·1 ml. 0·05M-$K_3Fe(CN)_6$
* { 2·0 ml. 2N-HCl 1·5 ml. 9N-HCl	* { 2·0 ml. 2N-HCl 1·5 ml. 9N-HCl
2·0 ml. fresh naphthoresorcinol solution (see above)	2·0 ml. fresh naphthoresorcinol solution

The contents of the tubes were then thoroughly mixed and the colour developed, extracted and estimated as described below for urine extracts.

Reproducibility. With the above method, a series of eight results with 0·15 mg. of Na*PG*(V) gave (colorimetric readings; Ilford 404 green filter): average E, 0·3254, σ of an individual reading, ± 0·0036 ('blank', 0·03). Eight results with 0·1 mg. of Na*PG*(V) as 'standard' at different times gave (difference between 'test' and 'blank' colorimeter readings; Ilford spectrum yellow filter): average E, 0·2795, σ of an individual reading, ± 0·0076.

Proportionality. Beer's law was fairly closely obeyed between 0·05 and 0·25 mg. Na*PG*(V) (0·04–0·20 mg. Na*PG*). Smaller amounts were not examined, and above 0·2 mg. Na*PG*, the colour increased only slightly. Fig. 1 shows results of a proportionality experiment.

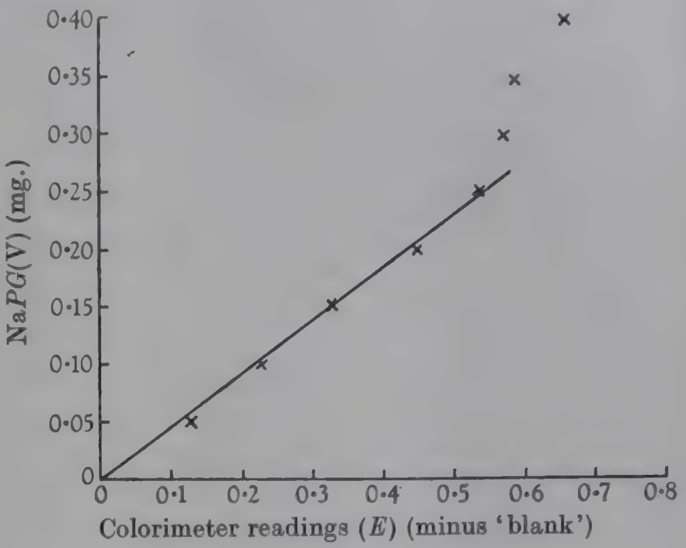

Fig. 1. Relationship between colorimeter readings and Na*PG*(V) estimated. Ilford 404 green filter.

The slight deviation from proportionality at the lower values we believe to be due largely to the impossibility of controlling the heating conditions (cf. Mills, 1946). According to Hanson *et al.* (1944) hydrolysis and colour development (in approx. 4·5N-HCl) may not be complete for 4 hr. and we do not believe that the reaction in our conditions (2·5N-HCl) is complete in 2·5 hr. However, the use of the reduced time does not appear to introduce a serious error, except perhaps when an artificial standard is used (see below). Solutions found to give colorimetric readings above the figure for 0·2 mg. Na*PG* (0·25 mg. Na*PG*(V)) were suitably diluted and re-estimated.

Entrainment of glucuronidate. In preliminary experiments we found that pregnanediol glucuronidate could be entrained on precipitates of $BaSO_4$ or of barium phosphate and, to a lesser extent, on other precipitates. Entrainment did not occur effectively at concentrations of sodium pregnanediol glucuronidate below about 5 mg./100 ml. and the process was, therefore, not directly applicable to urine; however, we decided to apply it to urine concentrates made with butanol. Barium phosphate was used as the entraining precipitate because it could be dissolved in the HCl used for the naphthoresorcinol reaction. Entrainment from 2 ml. of aqueous solutions of Na*PG* diluted with 2 ml. of water was carried out exactly as described for urine extracts (below): the precipitates were not washed.

Losses on entrainment. These are shown in Table 1. The results are possibly not inconsistent with the assumption that there is a loss (perhaps due to solubility) of an almost constant amount of glucuronidate on entrainment. A figure which we used in later experiments (see below) was 0·05 mg. of Na*PG*(V).

Washing of precipitates. To test the washing procedure used for precipitates from urine extracts, glucuronidate was entrained on barium phosphate as described for urine (below) and the precipitates, drained from supernatant liquid, were washed as described for those from urine extracts. Losses on washing by this method are shown in Table 2.

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* 3·5 ml. of 5N-HCl could be used: we used acid of the two strengths because we had to do so in entrainment experiments and wished to test the procedure.

Table 1. *Losses on entrainment of sodium pregnanediol glucuronidate*

(Results expressed as pure Na*PG*.)

Na*PG* entrained (mg.)	Av. loss (%)	σ of an individual % loss	No. of determinations
(*a*) Losses on entrainment from 2 ml. of Na*PG*(V) solution diluted with 2 ml. of water			
0·39	10·3	±4·4	6
0·31	15·9	±3·2	6
0·23	14·3	±3·4	6
0·16	20·6	±4·2	5
0·08	37·2	±8·3	6
(*b*) Losses on entrainment from 2 ml. of Na*PG*(M) solution diluted with 2 ml. of water			
0·36	11·2	±3·5	6
0·18	13·9	±2·3	6

Table 2. *Losses on washing entrained glucuronidate (from* Na*PG*(V))

Amount entrained (as mg. Na*PG*)	Av. % lost on washing	σ of an individual % loss	No. of determinations
0·31	1·0	±0·45	12
0·23	1·3	±0·73	12
0·16	4·5	±0·73	12
0·08	8·0	±1·94	12

The actual losses varied between 0·003 and 0·007 mg., as Na*PG*.

Extraction of Na*PG*(V) *with butanol.* Portions of 4 ml. each of a solution of Na*PG*(V) (50 mg./100 ml.) were added to 250 ml. of water containing 5 g. zinc acetate. The mixture was extracted with butanol, and the butanol was washed and evaporated to dryness as described for urine extracts (below).

The residue was heated almost to boiling with 10 ml. of water, and the cooled extract filtered; 2 ml. of the filtrate were diluted to 4 ml. and the estimation of glucuronidate (entrained but not washed) carried out as described for urine extracts (below). In two experiments losses were as follows (entrainment losses subtracted): Na*PG*(V) (as mg. Na*PG*) added to 250 ml. water, 1·55, 1·55; extraction loss, 15·5, 17·5%.

Preservation of urine. We found this most important in the estimation of *PLG* output (see, especially, Bucher & Geschickter, 1941). For example, in one series of 24 hr. collections, unpreserved urine, kept cold, was found to contain no measurable *PLG*, although the final (night) specimen had been added not more than 6 hr. before analysis. After experimenting, we finally added 3 g. of a 2 : 1 (w/w) mixture of finely powdered thymol and salicylic acid to the Winchester bottle in which absolutely fresh urine specimens were collected. Such urine (10 samples) showed no visible growth of micro-organisms after 48 hr. at 37°. The *PLG* content of preserved urine, kept at 37°, from a subject in the 9th month of pregnancy, did not fall until after 48 hr. As a routine, we kept urines, preserved as stated, at 0–5° prior to the analysis, which was in any case carried out as soon as was practicable.

Preliminary treatment of urines. To 300 ml. of urine we added 6 g. of zinc acetate. The mixture was shaken and filtered. This process made subsequent butanol extraction easier, and did not appear to affect the results of *PLG* determinations on urine from non-pregnant women. Without exhaustive experiments, one might hesitate to apply it to urines of very high *PLG* contents because of possible losses in the precipitate produced with zinc acetate: in a few experiments we have not observed such losses.

Estimation of PLG in urine. Urine (300 ml.), preserved as above, was treated with zinc acetate (6 g.), and the mixture was shaken and filtered; 250 ml. of the filtrate were mixed by rotation in a separating funnel with successive lots of 100, 50 and 50 ml. of purified butanol. Complete clearing of the lower layer was not awaited longer than *c.* 5 min. The combined butanol extracts were washed by rotating with 100 ml. of 0·2N-NaOH and then with two separate lots of 20 ml. of NaCl solution (2%, w/v): separation of layers was rapid and apparently complete. The butanol was now evaporated at 50–55°/*c.* 20 mm. and the residue finally dried by evaporation with ethanol (dried over CaO) *in vacuo*. The residue was dissolved in one of two ways: either (*a*) it was heated almost to boiling with $(10 \times 1500/x)$ ml. of water (where x ml. was the 24 hr. volume of the urine), or 10 ml. of water where the 24 hr. volume was unknown or unimportant; or (*b*) the residue was dissolved in 2 ml. of N-NaOH in the cold, followed by 2 or 3 ml. water; phenolphthalein indicator was added, and such quantities of N and then of 0·1N-HCl as were required to discharge the pink colour, water being finally added to make the final volume $(10 \times 1500/x)$ or 10 ml. as above.

The butanol residue from urine extracts contained a certain amount of acidic gums, as well as preservative, and it proved difficult to dissolve completely all the glucuronidate it contained unless heating or alkali were employed. It was also very important that the final aqueous extract of the residue should not be so alkaline as to produce a precipitate when $BaCl_2$ was added (see below), for such a precipitate would entrain extra, and uncontrollable, amounts of glucuronidate. The effect of making the final volume $(10 \times 1500/x)$ ml. was to 'correct' to a standard 24 hr. volume of 1500 ml. of urine, and so to obtain successive urine extracts in approximately the same concentration, whatever the actual 24 hr. volume: since *PLG* entrained depends partly on concentration (see below), we considered this manœuvre to be important. The solution of the residue, made as above, was filtered. A 2 ml. portion of the filtrate (*F*), in a 15 ml. conical centrifuge tube, was mixed with 2 ml. of water. The solution was treated with 1 ml. of 0·5M-$BaCl_2$, followed, after it had been cooled for 10 min. in ice, by 1 ml. of Na_2HPO_4 (0·05M, containing 50 ml. of N-NaOH/litre).

The tube was stoppered, the contents well mixed and again allowed to stand for 10 min. at 0°. Acetone (0·5 ml.) was used to wash the stopper and sides of the tube, so as to form an upper layer. The tube was then centrifuged at 2300 r.p.m. (radius of centrifuge head, 16·2 cm.) for 10 min. The supernatant fluid was poured off and the tube drained by inversion on filter paper. Acetone (2 ml.) (previously distilled over CaO) was added and stirred up with the precipitate with a fine wire; the tube was centrifuged for 10 min., the supernatant fluid discarded and the precipitate drained as before. The tube was then washed out with 10 ml. of saturated barium phosphate solution (added from a pipette *without* disturbing the precipitate), centrifuged for 5 min. and drained as before: this last washing was repeated. Finally, the precipitate was dissolved in 4 ml. of 2N-HCl. 'Blank' barium phosphate precipitates (from 4 ml. of water, then $BaCl_2$ and Na_2HPO_4 as above) were made and centrifuged: these were not washed; they were dissolved in 4 ml. of 2N-HCl.

Wide test tubes (15 × 2 cm.) were now prepared as follows:

Test (duplicated)	Standard (duplicated)	Blank
0·5, 1·0 or 2·0 ml. of HCl solution of ppt. and 2N-HCl to make 2 ml.	0·2 or 0·3 ml. Na*PG*(V) solution (50 mg./100 ml.)	—
1·4 ml. water	1·2 or 1·1 ml. water	1·4 ml. water
—	2·0 ml. 2N-HCl solution of 'blank' barium phosphate ppt. made as above	2·0 ml. 2N-HCl solution of 'blank' barium phosphate ppt. made as above

(All tubes) 1·5 ml. 9N-HCl, 0·1 ml. 0·05M-$K_3Fe(CN)_6$ and 2·0 ml. naphthoresorcinol reagent (above)

The contents of each tube were thoroughly mixed and small funnels were placed in the mouths of the tubes, which were suspended by rubber bands clear of the bottom of a water bath. The water was heated to 100° for 2·5 hr., the contents of each tube being again mixed shortly after heating had begun. The tubes were now cooled in ice and the contents of each in turn poured and washed into a small separating funnel with 14 ml. of ethanolic ether (20% (v/v) ethanol/peroxide-free ether). The stoppered funnel was vigorously shaken and, after separation, the aqueous portion of the contents run off. The coloured extracts were poured as quickly as possible from the mouth of the funnel into colorimeter tubes, which were immediately stoppered. The colours, which were stable for some time in absence of strong daylight (see below), were measured in a King (1946) type of single-cell photoelectric colorimeter (Gambrell Ltd.) with an Ilford 404 green or Spectrum yellow filter. The 'blank' reading was subtracted from each of the others.

Calculation. Since the standard contained 0·15 or 0·1 mg. Na*PG*(V), the concentration of *PLG* (as Na*PG*(V)) was

$$\frac{\text{reading of 'test' (minus 'blank')}}{\text{reading of standard (minus 'blank')}} \times 0{\cdot}15 \text{ or } 0{\cdot}1 \text{ mg. in } 0{\cdot}5,$$

1·0 or 2·0 ml. of HCl solution of barium phosphate precipitate. Hence the amount present in 4 ml. of HCl solution, i.e. entrained on to the precipitate from a known volume of urine, was calculated. Corrections for entrainment, washing, or butanol-extraction losses could not be applied, because of ignorance of the chemical nature and physical properties of *PLG*. The figures, as Na*PG*(V), could be corrected by reference to Na*PG*(M) and expressed as Na*PG* or pregnanediol (see above).

For urines very rich in *PLG*, e.g. those of late pregnancy, smaller amounts could be extracted and time thus saved in butanol evaporation. For some experiments, we were able to use 30 ml. of pregnancy urine, with one-tenth of the quantities of butanol, etc., for the extraction and washing process: the results agreed with those of larger-scale experiments.

With such urines, quantities of less than 2 ml. of the filtered aqueous extract (*F*) of the butanol residue could be diluted to 4 ml. with water, before entrainment, or amounts of less than 0·5 ml. of HCl solution of barium phosphate precipitate could be used for the estimation.

When larger amounts than 2 ml. of the filtrate (*F*) were used for entrainment, much higher results for *PLG* were often obtained (see under RESULTS). We do not consider that the extra glucuronidate thus entrained is all '*PLG*'.

Use of an artificial standard. In view of the difficulty of obtaining a suitable glucuronide of known purity as a standard, an artificial standard would clearly be of great value in the naphthoresorcinol reaction. Examination of the final coloured extract, prepared under our conditions, with a Hilger-Nutting spectrophotometer showed that the extract, provided it was kept in shadow, had almost constant absorption between 560 and 600 mμ., although its colour altered on exposure to strong daylight. It therefore seemed possible to use the grey solution of Thomson (1946) as a standard, with the Ilford Spectrum yellow light filter. Thomson's solution was diluted to twice its volume with water and in 17 separate experiments the ratio

$$\frac{\text{grey solution (colorimeter readings)}}{0{\cdot}078 \text{ mg. Na}PG \text{ (colorimeter readings } less \text{ 'blank')}}$$

gave, average, 1·24, σ of an individual reading, ±0·06. In 16 determinations, the ratio grey solution/0·117 mg. Na*PG* was, average, 0·92, σ of an individual reading, ±0·03.

For clinical use the method could probably be shortened, with some loss of accuracy, by reducing the time of heating in the naphthoresorcinol reaction to 0·5 hr. In this case some preparation of Na*PG* would have to be used as a standard, since the factors quoted above for Thomson's grey solution are for a 2·5 hr. heating period. We have found Beer's law to be less accurately obeyed for a 0·5 hr. heating period, so that the Na*PG* standard would need to approximate to the 'test' in concentration. Collection of 24 hr. urine specimens could be avoided, in individuals whose creatinine output can be assumed constant, by expressing the *PLG* as mg./g. of creatinine excreted (Jayle & Libert, 1946).

RESULTS

Constancy of PLG estimations. The results of four sets, of three estimations each, by the above method on different (male) urines were (in mg. Na*PG*/l.):

	Set 1	Set 2	Set 3	Set 4
	3·50	3·12	3·73	7·59
	3·23	2·56	4·99	6·55
	2·86	2·84	5·26	6·62
Av.	3·20	2·84	4·66	6·92

Recovery of Na*PG*(V) *added to urine extracts.* (The results given below and later, except for the female cycle, were obtained with 'aged' naphthoresorcinol, without $K_3Fe(CN)_6$.) Na*PG*(V) solution was added to 2 ml. of the filtered aqueous solution (*F*) of the butanol residues from urines, prepared as above. *PLG* was entrained, washed and estimated as described. Standards were entrained (not washed) amounts of 0·4 mg. of Na*PG*(V); 0·05 mg. Na*PG*(V) was assumed as entrainment loss (see above). Results per 2 ml. of filtered aqueous solution (*F*) of butanol residue, were:

Na*PG*(V) added (mg.) (0·05 mg. entrainment loss subtracted)	*PLG* estimated (as mg. Na*PG*(V))	Na*PG*(V) recovered (mg.)	Recovery (%)
0	0·350	—	—
0·150	0·489	0·139	92·6
0·350	0·730	0·380	108·4

Recovery of Na*PG*(V) *added to urine*. Na*PG*(V) in solution (50 mg./100 ml.) was added to 300 ml. of female 'follicular' urine, preserved as described. The whole process of *PLG* estimation was carried out, using standards of 0·4 mg. of entrained Na*PG*(V) as above. Results are shown in Table 3.

Table 3. *Recovery of* Na*PG*(V) *added to 'follicular' urine*

Na*PG*(V) added to 250 ml. urine (16% butanol extraction loss and 0·05 mg. entrainment loss subtracted) (mg.)	*PLG* found (as mg. Na*PG*(V))	Na*PG*(V) recovered (mg.)	Recovery (%)
0	3·06	—	—
2·20	4·54	1·48	67·2
0	1·77	—	—
2·32	3·79	2·02	87·1
0	1·85	—	—
2·25	3·78	1·93	85·8
0	2·78	—	—
0·92	3·70	0·92	100·0
0	3·33	—	—
0·84	4·37	1·04	124·0

The recovery figures given in Table 3 are insufficient to form a reliable statistical basis for calculating the error of the whole method, and, in any case, they are somewhat unreal because of the unknown (and presumably variable) properties of *PLG*. However, they serve to show that the method should allow the estimation, with a moderate degree of accuracy, of an increase of output of Na*PG* of 2·5 mg./l. of urine (1·5 mg. pregnanediol/l.).

PLG in various urines. Some results are shown in Table 4.

PLG in the normal female. Fig. 2 shows the results of *PLG* estimation for more than a complete cycle in a woman (21 yr., recently married, nulliparous). Portions of 2 ml. of filtered extract (*F*) were used for entrainment.

Table 4. *PLG in human urine*

(x=ml. of filtered aqueous extract (*F*), of the final butanol residue used for entrainment.)

Urine	*PLG* (mg. Na*PG*/24 hr.) $x=2$	*PLG* (mg. Na*PG*/24 hr.) $x=4$
Pregnancy (9th month)	41·1	56·2
	38·1	66·6
Normal male	7·0	8·2
	6·0	13·2
	4·7	12·5
	5·0	14·3
Infant female (2·5 years)	1·8	2·6

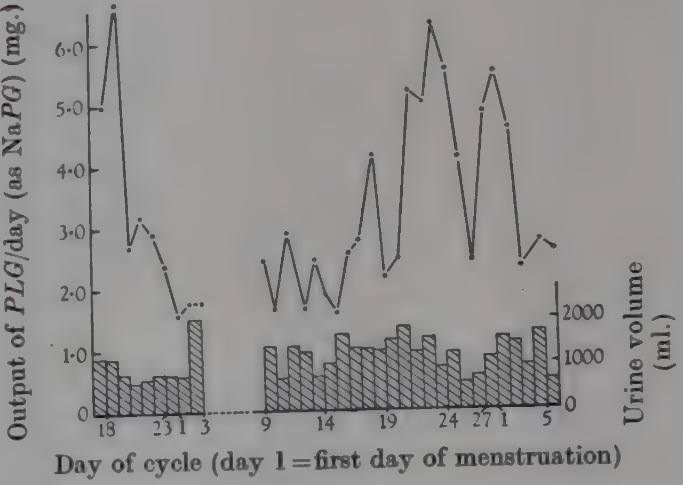

Fig. 2. *PLG* in female cycle.

All the above figures represent *minimal* values for *PLG*, because, as pointed out above, losses on butanol extraction and on entrainment cannot at present be estimated (except for Na*PG*) in the absence of precise knowledge of the properties of *PLG*.

DISCUSSION

The above results show that the method described will estimate Na*PG* with some accuracy at concentrations of about 2·5 mg./l. in urine in the presence of the rest of the *PLG* complex. The method, therefore, should be useful in the study of the human menstrual cycle, the 'luteal' phase of which we have had no difficulty in detecting, presumably because of the increased output of Na*PG* at this time. The method should also provide a rapid means of estimating *PLG* in late pregnancy, with small amounts of urine: further work would be needed to assess its value in early pregnancy. The conditions, other than pregnancy, in which *PLG* output may be increased include some forms of adrenal hypertrophy (cf. Venning, Weil & Browne, 1939; Salmon, Geist & Salmon, 1941) and, indeed, any condition in which excretion of steroids conjugated with glucuronic acid is augmented.

With regard to the use of the term 'pregnanediol-like glucuronide (*PLG*)', it must be emphasized that this is a very vague conception at present, and can only be given precise significance when the results of investigations into the chemical nature of urinary glucuronide are available. At present, the most that can be said is that *PLG* presumably contains at least part of the constituents of Na*PG*(V), which may include the sodium glucuronidates of pregnane-3(α)-ol-20-one (Marrian & Gough, 1946) and of pregnane-3(α):17:20-triol (Mason & Kepler, 1945) as well as of pregnane-3(α):20(α)-diol itself. It is of course possible that *PLG* may be found to include glucuronides not of a steroid nature, although this would be surprising in view of its solubilities. It is certainly clear that *PLG* from urine from normal males may contain very little Na*PG*. According to Westphal (1944), the amount of pregnanediol is about 0·7 mg./l. (as barium pregnanediol glucuronidate), and we, ourselves, have found only traces in our *PLG* complex from this source.

One can vary the fraction of *PLG* isolated by altering the conditions of extraction and especially of entrainment: with sufficiently 'drastic' entrainment conditions one presumably includes much more soluble glucuronidates, and possibly even barium glucuronate itself, in the estimations. With our conditions, however, we are of the opinion that the *PLG* estimated closely resembles Na*PG* in its solubilities and preliminary isolation experiments have shown that it gives a considerable ether-soluble fraction after hydrolysis. We were especially interested in the work of Jayle & Libert (1946), who have isolated 'steroid glucuronides' by two entirely different methods and estimated them by the naphthoresorcinol reaction. Their results resemble our own quite closely and Jayle & Libert appear also to be dealing with glucuronide fractions similar to that which we call '*PLG*'.

Finally, it may be pointed out that our entrained material may prove to contain other conjugates (e.g. organic sulphates) from urine, and may be useful for isolation of such substances.

The chemical nature of the glucuronide fraction is being investigated, but in the meantime the method is reported in the hope that it may be of value in the examination of urinary output of glucuronidate.

SUMMARY

1. A method of estimating pregnanediol glucuronidate by entrainment on barium phosphate and subsequent application of a modified naphthoresorcinol reaction has been developed.

2. The method when applied to urine gives analytical figures for a glucuronide fraction which we have called 'pregnanediol-like glucuronide (*PLG*)'.

3. Minimal figures for *PLG* (as Na*PG*) in various urines, including those of the human menstrual cycle, are reported.

4. The nature of *PLG* is discussed.

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Relations between Potassium and Sodium Levels in Mammalian Muscle and Blood Plasma

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In a previous account of the question of ionic permeability of the muscle fibre membrane (Conway, 1946), two regulative mechanisms were considered: one, a differential and passive permeability whereby potassium and chloride entered freely, whereas ions such as sodium, magnesium, sulphate, etc. were excluded (or entered relatively very slowly), such permeability being determined by the ion size; the other, a possible mechanism whereby sodium ions if they entered very slowly and over a long period could be extruded by some active process.

For the first of these mechanisms the evidence already presented (Boyle & Conway, 1941; Conway, 1946) scarcely admits of any other interpretation in the case of isolated sartorius of the frog, and applies also to the proximal convoluted tubules of the frog's kidney (Conway, FitzGerald & MacDougald, 1946) and liver (Conway & Boyle, 1944). Wilde (1945) has shown mammalian muscle to be permeable to chloride, and many studies with isotopic potassium (e.g. Noonan, Fenn & Haege, 1941) have shown mammalian muscle to be freely permeable to potassium ions. Also, Shanes (1946) has presented evidence showing that spider crab nerve is freely permeable to potassium and chloride.

Concerning the second mechanism, there is no satisfactory evidence that sodium ions which have entered into skeletal muscle fibres are actively extruded. The small amount of the exchanges recorded with respect to the muscle tissue as a whole leaves it open to reasonable doubt as to the exchange of the sodium ions from *within* the fibres. An outstanding experiment of Heppel (1939), working in Fenn's laboratory, showed that when rats were fed on a diet with very low potassium content, sodium undoubtedly entered the muscle fibres, but experiments demonstrating the reversal of the process and its rate do not appear to have been carried out, though the restoration of the potassium levels was demonstrated (Heppel & Schmidt, 1938). The importance of this question for muscle physiology and the theory of ionic exchanges across the membranes of mammalian tissue cells led us to repeat these experiments, and to find whether extrusion of sodium occurred on returning the rats to a diet rich in potassium. Although our findings confirmed those of Heppel, the amount of sodium entering did not occur to the same extent, due possibly to the shorter time allowed on the low potassium diet; the duration of Heppel's experiments was about 45 days, ours lasting upwards of 30 days on the low potassium intake. We found an average increase from 53 to 80 mg. sodium/100 g. muscle after 30 days, 53 mg./100 g. being an average normal figure for the group of rats used in these experiments. After 24 days the sodium increased to *c.* 76 mg./100 g. The corresponding chloride changes left no doubt that much the greater fraction of this sodium had entered the fibres. The changes with respect to sodium on restoring such rats to a high potassium diet, as well as the concomitant potassium, chloride and water changes, are described below.

METHODS

Experimental. Young rats, averaging about 70 g. in weight, of the Wistar strain were used throughout. They were maintained on the low K diet for the required number of days, after which they were bled into small dry centrifuge tubes and the serum separated immediately and stored for analysis. For the muscle analyses, a small portion of the leg muscle (about 100 mg.) was removed into a weighed platinum crucible for subsequent ashing and Na and K determinations; larger amounts were required in the series in which muscle Cl analyses were carried out.

Diet. The low K diet consisted of the following (given as percentages by weight): glucose 52, casein 30, salad oil 10, cod-liver oil 1·6, wheat germ oil 0·7, 'ammonia' yeast (yeast in which all the K had been replaced by NH_4 ions (Conway & Breen, 1945)) 1·6, and salt mixture 4·1. The salt mixture was similar in composition to that used by Heppel (1939). The K content of this diet was 7–10 mg./100 g. and the average daily consumption of the diet/rat was about 10 g. The high K restoration diet contained (in g./100 g.): wheat flour 36, oat flour 36, casein 5, yeast 21, $CaCO_3$ 1, NaCl 1, with 0·17 KCl added (total K = 340 mg./100 g.). For a group of rats maintained for 28 hr. on the extra high K intake, a similar diet was used with KCl added to form 1 g. KCl/100 g. (total K 780 mg./100 g.).

Chemical. Na and K were determined by colorimetric modifications (Boyle, Conway, Kane & O'Reilly, 1941) of the methods of Salit (1932) and of Shohl & Bennett (1928) respectively, after ashing as described by Fenn & Cobb (1934). Serum Cl was determined by a microdiffusion method (Conway, 1939; 1947) and muscle Cl by a similar method after a preliminary diffusion into isotonic Na_2SO_4. The water content was found by drying overnight at 105° prior to ashing for Na and K determinations.

RESULTS

The mean results for the first series are shown in Table 1. It will be seen that an increase of Na in skeletal muscle occurs as in the experiments of Heppel. The increase is at first rapid and then more gradual, reaching approximately 36·5 mmol./kg. after 26 days. Prolongation of the dietary to 46 days, as for Heppel's rats, would probably give figures not far different from his. For the individual results there is a rather wide variability. Thus, after the 26th day, the three rats examined had 46·6, 34·4 and 28·7 mmol. Na/kg. Similar variability may be seen in the results of Heppel, who gives the pooled analyses from 2 or 3 rats; after 46 days the values ranged from 64·4 to 44·8 mmol. Na/kg. and the range for single rats would no doubt be larger.

Table 1. *Effect of low* K *diet on muscle* Na, K, Cl *and water content*

(Mean values are given.)

No. of rats	Days on low K diet	Muscle Na (mmol./kg.)	Muscle K (mmol./kg.)	Muscle Cl (mmol./kg.)	Muscle water (g./kg.)	Plasma Cl (mmol./kg.)
10	0	22·8	100·5	12·4	765	117·5
2	4	25·4	86·8	10·7	757	119
2	8	31·4	72·8	13·0	775	117
2	12	34·4	75·3	14·4	770	116
2	16	34·5	81·6	15·2	763	117
3	26	36·5	75·4	15·2	760	122

Table 2. *Changes of muscle and serum* Na *and* K *on transfer from low* K *diet to high* K *diet*

No. of rats	Days on low K diet	Muscle Na (mmol./kg.)	Muscle K (mmol./kg.)	Serum Na (mmol./kg.)	Serum K (mmol./kg.)	Muscle water (g./kg.)
10	0	22·8	100·5	141	4·9	765
15	27	33·2	82·4	142	3·6	760
	Days after change to high K diet					
6	1	33·5	102·6	143	5·5	—
5	2	33·1	101·0	146	6·9	—
5	3	26·5	98·2	148	7·6	—
5	4	26·5	100·7	146	8·0	—
5	6	23·9	105·1	144	8·7	—
5	16	21·7	102·0	144	9·0	—

The mean increase in the muscle Na was from 22·8 to 36·5 mmol./kg. whereas the Cl increased from 12·4 to 15·2 or an increase of 2·8 mmol./kg. Only a small fraction of the Na increase could therefore be interpreted as due to change in the interspaces between the muscle fibres; in short, the Na must have largely entered the fibres.

Another point of interest from Table 1 is that there is no immediate relation between the rise of Na in muscle and fall of K. After 26 days the K reduction is about 25 mmol. whereas the Na increase is only about 14. After 16 days the fall in K is 19 whereas the Na increase is only 12 mmol./kg.

In the second series of experiments conducted very shortly after the first and on similar rats, a group of 50 rats was placed on the low K diet as before. For this group, the 1 % NaCl was replaced by a salt mixture similar to Heppel's. The rats were maintained on this low K diet for 27 days, then 15 were taken and the leg muscles and serum analyzed. The remaining rats were placed on the K-rich diet (containing 340 mg. K/100 g.) of which they partook avidly. Table 2 shows that the muscle Na falls slowly to normal over a period of about 16 days, and then slightly below normal. After 24 hr., though the plasma K is well above the normal value and the muscle K restored, there is no significant change in the muscle Na. The same holds after the second 24 hr., the plasma K being now as high as 6·9 mmol./l. It is only after 3 days that a significant decrease is observed. After 6 days the muscle Na is near to the normal average value and after 16 days slightly below this level.

It was of interest to find if very high levels of K, rapidly produced, would increase the rate of Na return from the muscle. Some rats of the above group after the 27th day were fed on a diet with the K content raised by further KCl addition to 780 mg./kg. The rats ate considerable amounts of this food and after 28 hr. the plasma K was as high as 9·8 mmol./kg. The mean Na content of the muscles of these rats was 28·2 mmol./kg., which was still much higher than the mean 22·8 of the normal group, but lower than the corresponding 33·5 of the rats having 5·5 mmol. K/l. of plasma. It would appear, therefore, that for mammalian muscle very high plasma K had some influence in hastening the return of muscle Na from mammalian muscle.

With amphibian muscle at room temperature or in the cold, no evidence of the return of muscle Na, much increased by previous immersions in K-free Ringer, could be obtained when using high external K.

DISCUSSION

From the above results the following conclusions may be drawn.

(1) Mammalian muscle has the power of slowly extruding Na from within the muscle fibres.

(2) The extrusion of the Na is comparatively very slow compared with the rates of K entrance. Thus, from the work of Noonan *et al.* (1941), labelled K enters to half its equilibrium value in about 45 min. The return of the muscle Na when the plasma K is increased beyond the normal level becomes noticeable after about 3 days, and after about 1 day when the K is raised considerably.

(3) The process of the restoration cannot be interpreted as an antagonism between Na and K or a competition for acidic groups on the non-diffusible constituents, since almost immediately the muscle K was restored to its old level and the plasma K became even higher than normal, whereas for 48 hr. in the above experiments no sign of Na decrease occurred. The explanation must take account of the relative rates of passage of the ions to and from the fibres, and of a membrane alteration produced by very low K levels.

An interpretation of the ionic permeability of the muscle-fibre membrane in relation to the experimental findings

This is essentially that already put forward (Conway, 1946) in which two mechanisms controlling the normal K and Na entrance are postulated. The first, a permeability such that while K and Cl ions enter rapidly, Na enters comparatively very slowly, possibly many hundreds of times more slowly than K. The second, a slow, active extrusion of the very slowly entering Na ions. This interpretation is not inconsistent with the previous treatment of the physiological significance of inorganic levels in the internal medium (Conway, 1945).

It is further considered that when the plasma K falls below the normal level, the membrane is placed under increasing strain owing to the very high potential gradient across it. This strain may be likened to the dielectric strain of a condenser. As a result, Na ions enter the muscle more rapidly than usual. The question of such a change of permeability arising out of potential gradients across the membrane increased above a critical value has also been considered by Wilbrandt (1947) in a recent review. If we now consider an extrusion mechanism which, up to a point, returns Na ions to the plasma at a rate largely proportional to their concentration, then a steady state will develop with each level of plasma K. At normal K levels, the Na content of the muscle will be low, and when the plasma K falls to about half its normal level it will be relatively high. Similarly, with high plasma K the muscle Na, though low in concentration, will fall still lower, and this fall may be measurable. On such a view the Na extrusion rate need not alter, but only the entrance rate; at the same time the extrusion rate may alter considerably with the increased permeability of the membrane. The fact that in the restoration process the lowering of the high Na content of the muscle fibres is slow compared with the rate indicated by Heppel's experiments with radioactive Na, suggests either a marked reduction of the exit rate of Na on increasing the plasma K or a slow restoration of the membrane abnormality. It is also possible that the rate of mixing with the labelled ions does not measure the rate of ion transport (Ussing, 1947).

The entrance rate of sodium into the fibres in the low plasma potassium experiments of Heppel, compared with the normal entrance rate of potassium

Heppel found that radioactive Na interchanged freely with the Na which had entered the fibres. The figures suggested an even faster entrance rate than the normal K value, and was so referred to by Krogh (1946). This suggestion of a faster rate than K, however, is not borne out by closer examination, for it must be considered that Na is entering from a high external Na level into a relatively low concentration, whereas on the other hand K is entering from a very low external level into a high concentration. When these facts are taken into account, Na may be shown to enter the fibres under the abnormal conditions at only about one thirtieth the rate of the normal K entrance, as derived from the data of Noonan *et al.* (1941). How the K rate is affected by the abnormally low plasma K has not been shown, but we may suppose that it does not remain unaltered and is almost certainly increased, and possibly very much increased.

The relative rates as indicated above may be derived as follows: The rate (dQ_{mr}/dt) at which labelled K after injection accumulates in a muscle fibre may be expressed by the equation

$$\frac{dQ_{mr}}{dt} = (P_p C_{pr} - P_m C_{mr}) \times \text{surface}, \qquad (1)$$

where P_r and P_m are the entrance and exit rates respectively of K for the muscle fibre (per unit concentration and unit surface) under the given conditions, and C_{pr} and C_{mr} are labelled K concentrations in plasma and muscle respectively. For the relatively short half period for equilibration the potential across the membrane may be taken as constant. Also, it may be noted that it has been shown (Boyle & Conway, 1941; Hill & Kupalow, 1930) that all, or practically all, the K in the muscle fibre is ionized and not bound to protein, so that the difference between P_r and P_m may be held to reflect only the effect of the potential difference within and without the fibre. To bring these P_r and P_m values more into relation with permeability figures as ordinarily expressed, and to Fick diffusion constants, it may be assumed that the diffusion from the outer to the inner medium occurs into zero concentration, and similarly for the inner to outer diffusion. In the steady state dQ_{mr}/dt is zero.

If the labelled K in the plasma is constant or nearly so during the half period of equilibration, it may be deduced from equation 1 that

$$P_p = \frac{0{\cdot}276r}{t_{0\cdot5}} \times \frac{C_m}{C_p}, \tag{2}$$

where r is the radius of the fibre (0·8 of which is present as 'fibre water') and $t_{0\cdot5}$ is the half period of equilibration. Writing a similar equation for labelled Na under the same conditions and dividing we obtain

$$P_p/P'_p = \frac{t'_{0\cdot5}}{t_{0\cdot5}} \times \frac{C'_p}{C'_m} \times \frac{C_m}{C_p} \tag{3}$$

in which the dashed symbols represent the values for Na. If we compare the entrance rate of labelled Na in Heppel's experiments with the entrance rate of labelled K into normal rats, then the concentration ratio of K in the fibre to K in the plasma is practically the same (in Heppel's data) as the normal ratio, and the potential difference also may thus be taken as the same. In such experiments of Heppel, the Na very probably enters at a more rapid rate than for normal fibres, seeing that Na has entered the fibres to a considerable extent, but if we suppose it to enter at the same rate, then the conditions of comparison would be the same or very similar.

Allowing for the interspace Na, it appears from Heppel's data that the half period of exchange of the labelled Na is approximately 15 min. Throughout this period the serum counts for the labelled Na do not change appreciably with time. From Noonan, Fenn & Haege's data the half period of the labelled K entrance in normal rats is of the order of 30–60 min. 45 min. may be taken as probably not far from the real figure, and it would appear from the data as a whole that there was no considerable fall in the labelled K in the plasma, the radioactive material being injected mainly intraperitoneally and for some observations, subcutaneously. It may be assumed then that the ratio of the half periods for the labelled Na equilibration and that of labelled K in such experiments is about three to one, so that

$$P_p/P'_p = 82/3 = 27,$$

i.e. the entrance rate of Na ions into the muscle under strain with the abnormally low plasma K is only about one thirtieth the rate of the entrance of K ions into the muscle under normal conditions. It may therefore be concluded that the entrance rate of Na ions into mammalian muscle in the living animal, and under conditions of membrane strain, is considerably less than the entrance rate of K ions, and for muscle under normal conditions it may be only a very minute fraction.

It would seem rational to suppose that a regulating system for the muscle fibre which would allow K ions to pass freely, but exclude Na ions without energy expenditure, would be more advantageous than one requiring energy for the extrusion of the Na ions which freely entered. The formation of a membrane by the cell or muscle fibre which would achieve this *perfectly*, and at 37°, is perhaps impossible, but it would appear that a close approximation to it can be achieved, whereby the energy requirement for Na extrusion approaches near to zero. It may also be added that in numerous experiments we have been unable to detect any extrusion rate of Na ions from the frog's sartorius in the cold.

SUMMARY

1. Sodium which had entered the muscle fibres of rats, after a period of potassium deprivation, was slowly extruded when the rats were placed on a diet rich in potassium. The mean half period of extrusion, even with the plasma potassium above the normal level after 24 hr., was about 3 days.

2. The muscle potassium, which had fallen to levels of 75–82 mmol./kg. in the average, was fully restored to normal values (about 100 mmol./kg.) after 1 day on the high potassium diet, and did not appreciably change from this when the plasma concentration reached 9·0 mmol./kg.

3. Considerable variability was shown by individual rats in the amounts of sodium which had entered the muscle fibres during potassium deprivation.

4. Examination of the data of Heppel (1940) for the rate of mixing of labelled ions with the sodium which had entered the fibres during the low potassium period shows that the true entrance rate of potassium/unit concentration into such fibres is about 30 times as great as that of sodium.

5. The relative rate of passage of potassium ions into the normal fibres may be far greater than this, as evidenced by the comparatively very slow extrusion of muscle sodium, and it is also possible, as suggested by Ussing (1947), that the rate of mixing of the labelled ions may give values which are too high for ion transport.

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The Microbiological Degradation of Steroids

4. FISSION OF THE STEROID MOLECULE

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During recent years the presence of an enzyme system capable of effecting the oxidation of hydroxysteroids to the corresponding keto derivatives has been clearly demonstrated in a number of bacterial species. The investigations of the Italian schools of Ercoli and Arnaudi have resulted in the isolation from yeast of several new organisms, *Flavobacterium dehydrogenans* (Arnaudi, 1939), *F. androstenedionicum* (Ercoli & Molina, 1944), and *F. carbonilicum* (Molina & Ercoli, 1944), which, in general, oxidize the nuclear hydroxyl groups of steroid compounds devoid of the 17 side chain. Zimmermann & May (1944) obtained similar oxidations by the action of pseudodiphtheria bacilli. In the bile acid series stepwise oxidation of the hydroxyl groups has been achieved using *Alcaligenes faecalis* (Schmidt, Hughes, Green & Cooper, 1942), and more recently, *Escherichia coli* (Schmidt & Hughes, 1944). The independent investigations of the flora of soils by Tak (1942), and by the present author (Turfitt, 1943, 1944*a*), have shown that the cholesterol-decomposing organisms fall essentially within the genus *Proactinomyces*. These organisms are of particular interest in the breakdown of steroids since their oxidizing action is not, as reported for other organisms, inhibited completely by the presence of the intact side chain. Their place in the economy of nature is discussed later.

Whilst this primary oxidation of steroid hydroxyl groups is firmly substantiated, actual breakdown of the steroid molecules, yielding distinct chemical fission products, has not hitherto been recorded. Evidence of a secondary action of this nature has, however, been obtained during large-scale work on the oxidation of cholesterol to cholest-4-en-3-one (Turfitt, 1944*b*). With cholesterol as the sole carbon source, energy for growth must inevitably, in view of the non-autotrophic character of *Proactinomyces*, have resulted from utilization of the sterol carbon. During the course of the work certain small fractions were obtained which undoubtedly represented products of degradation, but attempts to isolate definite compounds were unsuccessful. A detailed account of the various methods by which information has been gained as to the course of the degradation process is given in the present publication.

EXPERIMENTAL

Foci of bacterial attack

Fission of the steroid skeleton by bacterial oxidation might be expected, by analogy with chemical methods, to occur primarily at one of several susceptible points, viz. OH groups, double bonds, or the side chain. With a view to determining whether the presence or absence of each of these points exerts any material effect upon the bacterial oxidation, a series of compounds covering different combinations of OH, double bond and side chain has been subjected to the oxidizing action of *P. erythropolis*.

In each experiment, performed in triplicate, 50 mg. finely powdered, sterile compound were introduced with aseptic precautions into a 250 ml. conical culture flask containing 100 ml. mineral salt solution, previously sterilized at 115° for 15 min. (The mineral salt solution referred to throughout this publication had the following composition: NH_4NO_3, 0·1; K_2HPO_4, 0·025; $MgSO_4.7H_2O$, 0·025; NaCl, 0·0005; $FeSO_4.7H_2O$, 0·00001%.) Inoculation was performed from 24 hr. agar-slope cultures of *P. erythropolis* (strain 15/G), and the cultures incubated at 25°. At intervals of 4, 7 and 11 days test samples of the cultures were treated with Universal Indicator (British Drug Houses Ltd.), production of acid being taken as indicating steroid decomposition.

A summary of the results of these experiments is given in Table 1. With those compounds in which the side chain is shortened either partially or completely to >CO, degradation is apparently more rapidly initiated. When, on the other hand, the hydrocarbon side chain is intact, blocking of the hydroxyl group or of the double bond, or of both, does not prevent attack, although it seems to occasion some slight delay. Unless, therefore, there exists some other unsuspected point of oxidative attack, this would appear to be presumptive evidence of side-chain disruption.

Table 1. *Steroid utilization by* P. erythropolis

Compound	Points available			Universal indicator		
	—OH	Double bond	Side chain	4 days	7 days	11 days
Cholesterol	+	+	+	YR*	R	R
Stigmasterol	+	+	+	YR	R	R
Sitosterol	+	+	+	YR	R	R
Coprosterol	+	−	+	Y	R	R
Dihydrocholesterol	+	−	+	Y	R	R
Cholesterol acetate	−	+	+	YR	R	R
Coprosterol acetate	−	−	+	Y	R	R
Cholestane	−	−	+	Y	R	R
3-Hydroxychol-5-enic acid	+	+	+ (modified)	R	R	R
*trans*Dehydroandrosterone	+	+	−	R	R	R
Androsterone	+	−	−	R	R	R
Androsterone acetate	−	−	−	R	R	R

* R = Red (pH < 4); Y = Yellow (pH 6·5); reaction of original medium = Green (pH ~ 8).

Attempted isolation of volatile products

During the classical work on sterol structure two volatile materials were isolated which had resulted from oxidative disruption of the side chain, viz. acetone, originating from the terminal *iso*propyl group (Windaus & Neukirchen, 1919), and methyl *iso*hexyl ketone representing removal of the complete side chain (Windaus & Resau, 1913). Attention has thus first been directed towards determining whether the *Proactinomyces* oxidation proceeds along a similar course with the production of volatile ketones or possibly laevulaldehyde.

A 1 l. conical flask containing 250 ml. mineral salt solution and 2·5 g. $CaCO_3$ was autoclaved at 115° for 15 min., and after cooling 2 g. finely powdered sterile coprosterol acetate were added. The flask and contents were inoculated with *P. erythropolis* (strain 15/G) and incubated at 25° for 2 months, during which time sterile O_2 was slowly bubbled through the culture and thence through 25 ml. saturated solution of *o*-nitrobenzaldehyde in 2N-NaOH contained in a 100 ml. tube. At the end of 2 months the contents of the tube were warmed for 5 min. on the water bath, cooled and extracted with 5 ml. chloroform; the red or blue colour characteristic of the $CH_3.CO$— grouping under these conditions was not obtained. The experiment was repeated with like result using cholestane as substrate. Superficially this would appear to indicate that during this steroid decomposition volatile ketones and aldehydes are not produced, and in particular that the side chain is neither partially nor completely removed in the manner encountered in chemical oxidations. The results, however, are equally consistent with the theory that volatile compounds are produced, and are at once utilized by the organisms. In support of this theory it has been found by cultural experiments that *P. erythropolis* readily utilizes acetone, methyl *iso*hexyl ketone and laevulaldehyde when each of these substances constitutes the sole available carbon source in the culture medium.

Degradation of cholest-4-en-3-one

A quantitative study has been made of the production of cholest-4-en-3-one from cholesterol by *Proactinomyces* oxidation, and of its subsequent decomposition.

Six 250 ml. conical culture flasks, each containing 100 ml. mineral salt solution and 0·5 g. $CaCO_3$, were autoclaved at 115° for 15 min., and into each flask were introduced aseptically 50 mg. finely powdered cholesterol previously sterilized by steaming in a sealed tube. After inoculation from 24 hr. agar-slope cultures of *P. erythropolis* (strain 42/G), the flasks were incubated at 25° for periods of $\frac{1}{4}$, $\frac{1}{2}$, 1, 2, 3 and 4 months respectively. After the appropriate incubation period, each culture was removed and thoroughly extracted with chloroform; the extract was washed, dried with anhydrous Na_2SO_4 and evaporated. Curve *A* (Fig. 1) shows the decrease in total chloroform-soluble material throughout the culture period. The residues were redissolved in chloroform. Residual cholesterol (curve *B*) was determined by the method previously described (Turfitt, 1943), and 3-ketosteroids (curve *C*) by the Callow modification (Callow, Callow & Emmens, 1938) of the original Zimmermann procedure (Zimmermann, 1936). In order to determine decomposition products present as either soluble or insoluble Ca salts, the aqueous suspension from the initial chloroform extraction was acidified with diluted HCl, and re-extracted with chloroform; at no time, however, was more than 1·5 mg. residue obtained from the extracts. The total chloroform-soluble material was accounted for almost entirely by cholesterol + cholest-4-en-3-one, and it was evident that degradation products of cholest-4-en-3-one only existed in the cultures in minute amounts.

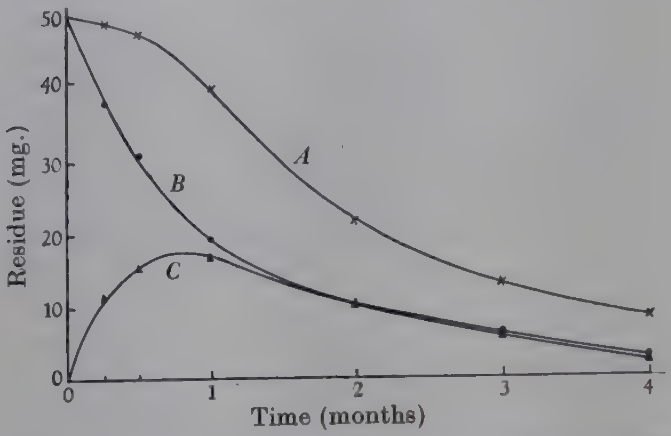


Fig. 1. Variation of total chloroform-soluble material (curve *A*), cholesterol (curve *B*), and 3-ketosteroids (curve *C*) in culture of *P. erythropolis* on cholesterol.

In an endeavour to isolate individual breakdown products in sufficient quantity for characterization, a considerable number of large-scale experiments was tried, each using 200 g. cholest-4-en-3-one prepared chemically from cholesterol by the Oppenauer oxidation (Oppenauer, 1941). In spite of the wide variety of experimental conditions no success was achieved, and it became increasingly apparent that the primary products of fission of the molecule were themselves attacked by the organisms in preference to the parent steroid, and that trapping of any acidic products as Ca salts did not prevent their further breakdown.

Continual removal of the breakdown products from the sphere of action of the organisms appeared to be the only logical method of isolating any intermediates. No suitable process for the purpose could be found recorded in the literature, and a technique was finally perfected employing the continuous culture apparatus shown in Fig. 2, which was kindly constructed to the author's specification by Messrs Quickfit and Quartz, King's Norton, Birmingham. Finely powdered sterile cholest-4-en-3-one (20 g.) was introduced into the previously autoclaved 2 l. reaction vessel *A*, which was then connected to the sterile unit *B* containing 3 l. mineral salt solution, and to the receiver units *U* and *C*. With the tap *T* open, culture medium was allowed to siphon into *A*, and a heavy inoculum of *P. erythropolis* (strain 42/G) was introduced. A fresh supply of sterile medium was connected to vessel *A*, and the tap adjusted so that the rate of flow from the nozzle *N* was approximately 1 drop/sec. Three litres of fresh culture medium were thus admitted during approx. 24 hr. Throughout the period of incubation (2 months) the reaction vessel was immersed in a water bath maintained at 25°. Outflow of medium occurred via the side arm *S* where loss of solid particles of steroid was prevented by the loose-fitting glass-wool plug. Sterile O_2 was passed continuously through the medium at the rate of approx. five bubbles/min., whilst the particles of the sediment at the bottom of the reaction vessel were gently agitated at intervals of a few hours. Further bacterial activity in the effluent was prevented by allowing the issuing drops to pass through a 1 in. layer of ether in the unit *U*, the ether being replenished from time to time to maintain this layer. The ether-saturated effluent was collected in the 3 l. flask *C*.

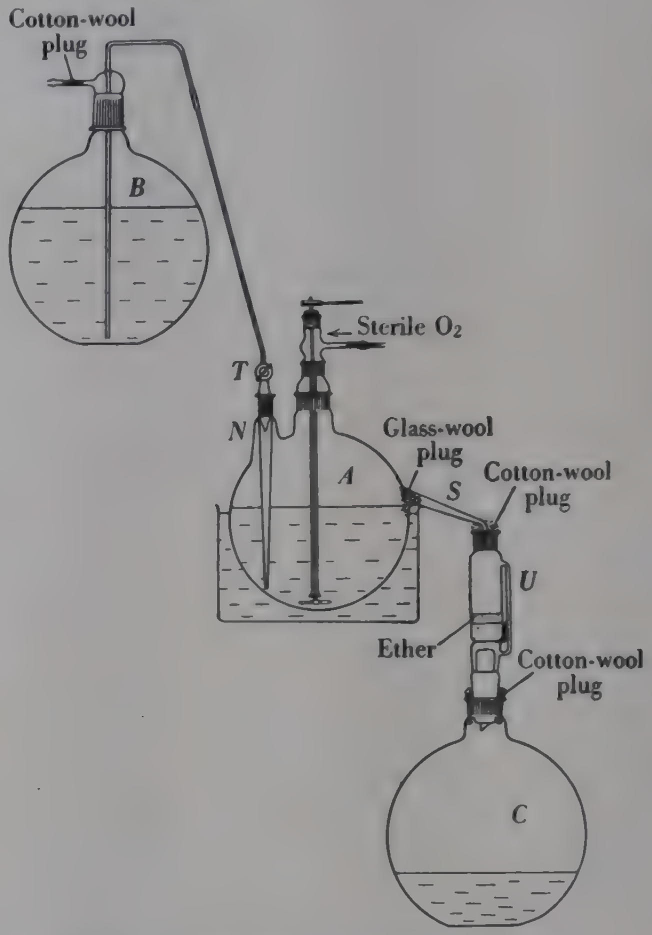


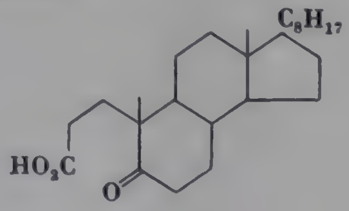
Fig. 2. Apparatus for continuous culture and collection of products.

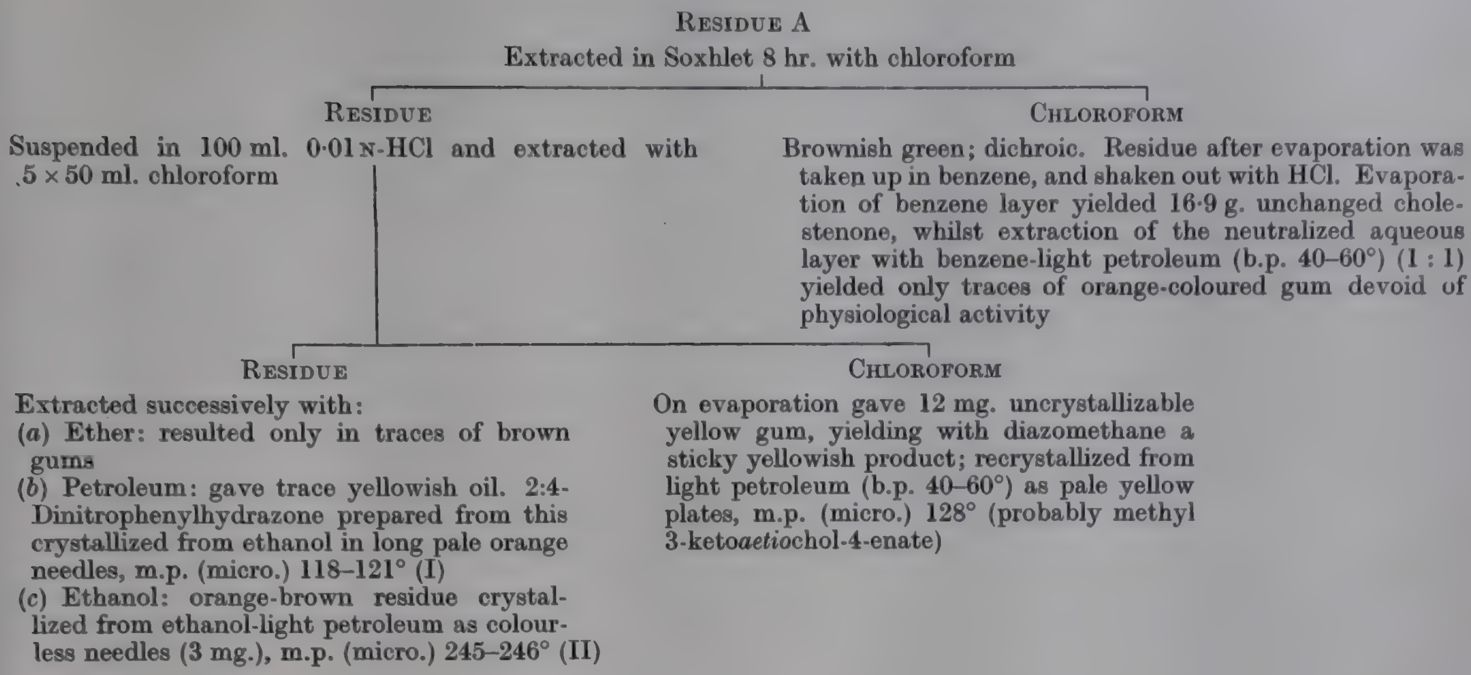
After 2 months' incubation the assembly was dismantled, the contents of the reaction vessel filtered, and the filtrate added to the pooled effluents; the residue (A) was dried in an air oven at 25°. By evaporation under reduced pressure of the ether-saturated spent medium, there were obtained 500 ml. of a pale straw-coloured liquid with a heavy granular deposit (B).

The detailed examination of the fractions A and B is given schematically in Figs. 3 and 4.

Characterization of products III and IV

Product III. On standing, the gum deposited rosettes of white needles. By repeated fractional crystallization from light petroleum (b.p. 100–120°) two products were obtained: (*a*) 4 mg. white plates, m.p. 150°. A specimen of the keto-acid of the formula





RESIDUE A

Extracted in Soxhlet 8 hr. with chloroform

RESIDUE

Suspended in 100 ml. 0·01 N-HCl and extracted with 5 × 50 ml. chloroform

CHLOROFORM

Brownish green; dichroic. Residue after evaporation was taken up in benzene, and shaken out with HCl. Evaporation of benzene layer yielded 16·9 g. unchanged cholestenone, whilst extraction of the neutralized aqueous layer with benzene-light petroleum (b.p. 40–60°) (1 : 1) yielded only traces of orange-coloured gum devoid of physiological activity

RESIDUE

Extracted successively with:

(*a*) Ether: resulted only in traces of brown gums

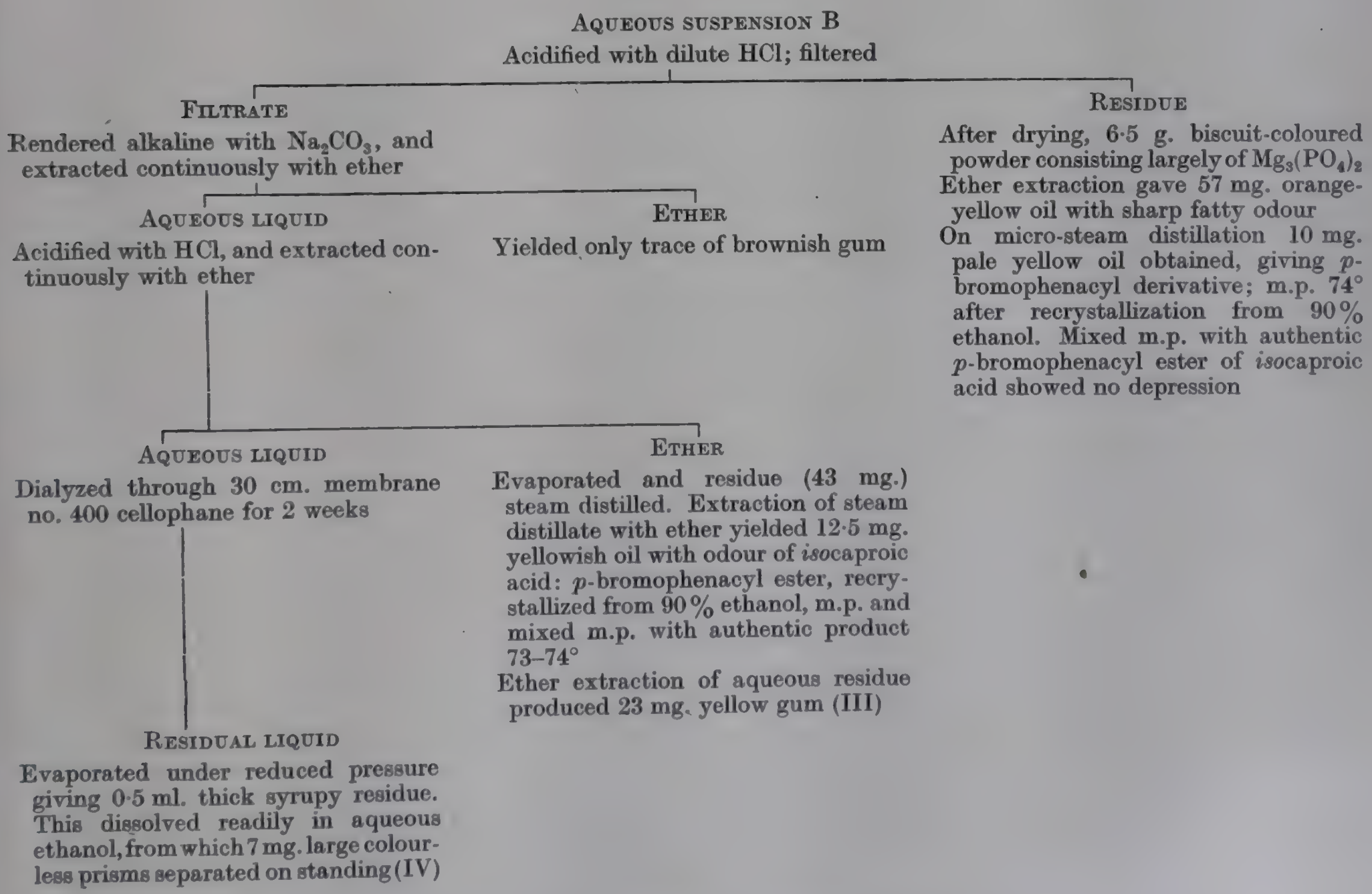
(*b*) Petroleum: gave trace yellowish oil. 2:4-Dinitrophenylhydrazone prepared from this crystallized from ethanol in long pale orange needles, m.p. (micro.) 118–121° (I)

(*c*) Ethanol: orange-brown residue crystallized from ethanol-light petroleum as colourless needles (3 mg.), m.p. (micro.) 245–246° (II)

CHLOROFORM

On evaporation gave 12 mg. uncrystallizable yellow gum, yielding with diazomethane a sticky yellowish product; recrystallized from light petroleum (b.p. 40–60°) as pale yellow plates, m.p. (micro.) 128° (probably methyl 3-keto*aetio*chol-4-enate)

Fig. 3. Fractionation of solid residue from degradation of cholest-4-en-3-one.



AQUEOUS SUSPENSION B

Acidified with dilute HCl; filtered

FILTRATE

Rendered alkaline with Na_2CO_3, and extracted continuously with ether

AQUEOUS LIQUID

Acidified with HCl, and extracted continuously with ether

ETHER

Yielded only trace of brownish gum

AQUEOUS LIQUID

Dialyzed through 30 cm. membrane no. 400 cellophane for 2 weeks

ETHER

Evaporated and residue (43 mg.) steam distilled. Extraction of steam distillate with ether yielded 12·5 mg. yellowish oil with odour of *iso*caproic acid: *p*-bromophenacyl ester, recrystallized from 90% ethanol, m.p. and mixed m.p. with authentic product 73–74°

Ether extraction of aqueous residue produced 23 mg. yellow gum (III)

RESIDUAL LIQUID

Evaporated under reduced pressure giving 0·5 ml. thick syrupy residue. This dissolved readily in aqueous ethanol, from which 7 mg. large colourless prisms separated on standing (IV)

RESIDUE

After drying, 6·5 g. biscuit-coloured powder consisting largely of $Mg_3(PO_4)_2$

Ether extraction gave 57 mg. orange-yellow oil with sharp fatty odour

On micro-steam distillation 10 mg. pale yellow oil obtained, giving *p*-bromophenacyl derivative; m.p. 74° after recrystallization from 90% ethanol. Mixed m.p. with authentic *p*-bromophenacyl ester of *iso*caproic acid showed no depression

Fig. 4. Fractionation of fluid product of degradation of cholest-4-en-3-one.

prepared by the original method of Windaus (1906) gave no depression of the melting point in admixture with this product. (*b*) 9 mg. slightly yellow crystals, m.p. 245°. Methyl derivative, prepared by means of diazomethane, and crystallized from light petroleum (b.p. 40–60°), had m.p. 130°, not depressed when mixed with methyl 3-keto-*aetio*chol-4-enate.

Product IV. The prisms, m.p. 96–98°, were readily soluble in water, the solution being neutral to litmus, slightly soluble in 90% ethanol, and insoluble in ether, chloroform and light petroleum. N, S, P and halogens were absent. On heating, the product melted and then charred with a 'burnt-sugar' odour. The solution did not reduce Fehling's solution, but gave a positive Molisch test. A red colour was obtained by heating with resorcinol in HCl, but no reaction was obtained with phloroglucinol in HCl. 2:4-Dinitrophenylhydrazine also gave a negative response.

Owing to the small yield of product no further attempts to characterize the material were possible. At present, speculation as to the origin of this product is somewhat premature, but if it should be found to be a genuine product of cholestenone degradation it would produce a most valuable clue to the ultimate fate of the ring structure. The likelihood is, however, that it is a product of synthesis, a view which would seem to be supported by the fact that carbohydrates are not in general susceptible to utilization by *Proactinomyces* spp.

The products isolated and characterized as a result of the above process demonstrate the course of the initial fission of the cholestenone molecule under *Proactinomyces* attack. *iso*Caproic acid and 3-keto-*aetio*chol-4-enic acid are obviously complementary products of the fission of the side chain at C_{20}–C_{22}, the 21–CH_3 being lost completely. This evidence of the removal of the entire side chain affords an explanation of the failure to isolate the intermediates, 3-ketochol-4-enic acid, 3-keto*nor*chol-4-enic acid and 3-keto*bisnor*chol-4-enic acid which would be expected to result from a stepwise breakdown of the side chain. A simultaneous rupture of ring *A* is indicated by the production of the keto acid already known to result from hypobromite oxidation of cholestenone. The extreme smallness of the yields fully justifies the theory that the initial breakdown products are themselves rapidly utilized, so that the unidentified materials (I) and (II) must be taken either to represent secondary degradation products, or to contain products derived from the simultaneous oxidations at the two susceptible points.

Degradation of 3-ketochol-4-enic acid

The use of 3-hydroxychol-4-enic acid as substrate in *Proactinomyces* cultures leads to a large and rapid increase in bacterial population, and the fall in pH of the medium occurs somewhat earlier than in the case of cholesterol. Since the structure of the primary oxidation product, 3-ketochol-4-enic acid, is essentially that of cholest-4-en-3-one, apart from the modified side chain, further oxidation should follow a similar course in both compounds.

A quantity of 10 g. of 3-ketochol-4-enic acid was treated in the continuous culture apparatus as described under cholestenone, and the 'spent' medium and residual solid substrate carried through an identical extraction process. A weight of 8·7 g. of unchanged 3-ketochol-4-enic acid was recovered, and 3-keto*aetio*chol-4-enic acid isolated in 7 mg. yield.

No conclusive evidence of a fission of ring *A* could be obtained, since the only other materials isolated consisted of uncrystallizable yellowish brown gums.

Degradation of cholesterol acetate

Previous experience with cholesterol acetate as substrate has been that, whilst the bacterial count increases and acid is produced, the steroid is largely recovered unchanged. From this it has been assumed that *Proactinomyces* possess no enzymic mechanism capable of hydrolyzing the esterified linkage, thus permitting oxidation of the liberated OH group.

A quantity of 20 g. cholesterol acetate has been treated according to the process described above, incubation in this instance being continued for a total period of 3 months. Even after this time 18·9 g. of the initial material were recovered unchanged. *iso*Caproic acid was obtained in 10 mg. yield, indicating a disruptive oxidation of the side chain, but repeated attempts to isolate a crystalline product from the 26 mg. of brownish gum expected to contain the acetate of 3-hydroxy*aetio*chol-5-enic acid were unsuccessful.

Since no evidence of any kind was obtained in this experiment of an actual ring fission, it still appears that in the case of cholesterol acetate there is but a single focus of initial bacterial attack.

Degradation of 3:7:12-*triketocholanic acid*

In the oxidation of cholic acid by *Alcaligenes faecalis* (Hoehn, Schmidt & Hughes, 1944) stepwise oxidation of the three hydroxyl groups occurred, the 3(α)-OH being oxidized last, and 3:7:12-triketocholanic acid was obtained as end product in 85% yield. The general method and detailed isolation technique introduced by these workers has been followed in an investigation of *Proactinomyces* attack on cholic acid, and essentially similar results have been obtained. The further, disruptive oxidation of 3:7:12-triketocholanic acid is of considerable interest, since each of the three keto groups should theoretically provide a susceptible point for bacterial attack, in addition to the probable attack on the shortened side chain. A complicating factor, however, from the cultural standpoint is the greater solubility of cholic acid and the fact that growth of *Proactinomyces* is inhibited in concentrations above approximately 1500 mg./100 ml.

The continuous culture process described in detail above was employed in slightly modified form. Initially, the empty reaction vessel *A* was autoclaved and connected with the units *B* and *UC*, 10 ml. water containing the combined surface growth of three 24 hr. agar-slope cultures of *P. erythropolis* (strain 42/G) being subsequently introduced into the reaction vessel. The flask *B* and contents, 3 l. mineral salt solution + 1·0 g. cholic acid, sterilized by autoclaving 5 min. at 115°, were replaced as required in order to maintain a continuous flow of solution at approx. 1 drop/sec. After 2 months' incubation, culture medium containing 52 g. cholic acid had been utilized. The spent medium, including the final contents of the reaction vessel, was evaporated under reduced pressure to 1 l. The resulting orange-coloured concentrate was rendered alkaline with ammonia and filtered. Solvent extraction of the residue (A) did not yield any identifiable product, neither was any material other than traces of brown gum obtained by continuous extractions of the residue, after suspension in dilute HCl, with light petroleum (b.p. 40–60°) and ether. The filtrate (B) was acidified with dilute HCl, and cooled with ice. On standing, the heavy, granular precipitate became crystalline, and was filtered off and dried (27·3 g.). After recrystallization from ethanol this product had m.p. 234°; in admixture with authentic 3:7:12-triketocholanic acid (m.p. 236°), a m.p. of 228° was obtained. The crystalline product was accordingly examined for the presence of intermediate oxidation products by the method described by Hoehn *et al.* (1944), but neither cholic acid nor any hydroxyketocholanic acid was isolated. The product was dissolved in 60 ml. methanol, 1 ml. conc. H_2SO_4 added, and the solution gently refluxed for 15 min. No precipitate was obtained by cooling in ice, but on addition of 10 ml. water followed by further ice cooling for 24 hr., a deposit of rosettes of colourless needles was obtained, m.p. 222°. Repeated recrystallization from aqueous ethanol only raised the m.p. to 228°, which was depressed to 218° in admixture with a specimen of methyl 3:7:12-triketocholanate. The product was hydrolyzed and the free acidic materials regenerated. Repeated fractional crystallization from ethanol-acetone-ether yielded 15·7 g. 3:7:12-triketocholanic acid, m.p. 234°, and 19 mg. of a crystalline acid, m.p. 240°. Further recrystallizations of this latter product from acetone-ether did not raise the melting point. In admixture with authentic 3:7:12-triketo*aetio*cholanic acid, m.p. 245°, no depression was observed.

The isolation of this product of degradation points to the side chain as the primary focus of attack on the molecule, and a careful search has failed to isolate any product arising from rupture of the ring system.

Oxidation of α-oestradiol

Proactinomyces oxidations in the steroid group have hitherto been concerned solely with compounds of saturated ring structure. In view of the readiness with which these organisms utilize simple aromatic compounds such as phenol and naphthalene (Gray & Thornton, 1928), it appeared probable that the benzenoid ring structure in steroid compounds would present no obstacle to *Proactinomyces* attack. In several small-scale experiments with *P. erythropolis*, however, whilst there was some slight increase in bacterial numbers, the pH of the medium remained unchanged, and the whole of the starting material was recovered unaltered. These unexpected initial failures were at first attributed to a diminution in the oxidative capacity of the particular strain used, but similar lack of success was encountered with typical strains of all the known *Proactinomyces* species. A more detailed study of the process was accordingly made as follows.

Finely ground sterile α-oestradiol (100 mg.) was introduced aseptically into 250 ml. sterile mineral salt solution contained in a 500 ml. conical culture flask. An extremely heavy inoculum, the growth from twelve 24 hr. agar-slope cultures of *P. erythropolis* (strain 143/N), was added. The culture was incubated at 25° for 2 months, during which time the flask was gently rocked, and a steady stream of sterile oxygen was bubbled through the culture. After the incubation period the medium was filtered, and the residue (A) dried in an air oven at 25°. The filtrate (B) was extracted successively with light petroleum (b.p. 40–60°), ether and chloroform, and this extraction was repeated after acidification of the aqueous liquid with dilute H_2SO_4; evaporation of the extracts yielded only minute amounts of brown resinous gums. The dried residue (A) was extracted continuously with chloroform in a Soxhlet for 3 hr., and the extract evaporated to dryness under reduced pressure. The residue was dissolved in 15 ml. 75% ethanol, and shaken with three successive 5 ml. quantities of light petroleum (b.p. 40–60°). The ethanol phase was separated, and examined for unchanged α-oestradiol and for oestrone by a modification of the technique described by Szego & Samuels (1943). On evaporation under reduced pressure 97·3 mg. almost colourless residue was obtained. This was dissolved in 20 ml. 15% acetone-light petroleum (b.p. 40–60°) and run on to a column (10 × 1 cm.) of 'adsorption alumina' (British Drug Houses Ltd.) previously treated with the same solvent. The column was washed with 80 ml. of acetone-light petroleum (b.p. 40–60°) mixture. The eluate was evaporated to dryness on a water bath, and the crystalline residue dissolved in 10 ml. ethanol, a separation of ketonic and non-ketonic materials being effected by the Girard (T) technique. The non-ketonic fraction consisted of 85·6 mg. almost pure α-oestradiol. Since the 5·5 mg. of brownish ketonic fraction could not be induced to crystallize an attempt was made to prepare a semicarbazone. 3·1 mg. long, colourless needles were thus obtained, and recrystallized from aqueous ethanol. No depression of the m.p. (256°, micro.) was observed in admixture with a prepared specimen of the semicarbazone of oestrone.

This experiment has been repeated with other *Proactinomyces* spp., but in no case has any actual degradation product been isolated, and oxidation at C_{17} with formation of oestrone is detectable only when the bacterial population is abnormally high. Ercoli (1941) has already reported a similar experience with *Micrococcus dehydrogenans*.

During this work on α-oestradiol the interesting observation was made that many *Proactinomyces* strains, after prolonged culture upon the diol as sole carbon source, show a diminution in their capacity for oxidizing cholesterol and other steroids with the intact 17 side chain; typical examples are given in Table 2.

There is no immediately apparent explanation of this phenomenon, and the fact that it is not

Table 2. *Diminution of steroid-decomposing capacity*

	Cholestenone from 100 mg. cholesterol; 2 months incubation	
Strain	Before oestradiol culture (mg.)	After oestradiol culture (mg.)
P. erythropolis M 14	20	17
P. erythropolis M 18/G	25	9
P. erythropolis 42/G	31	23
P. globerulum H/53	17	12
P. aquosus T 5/G	29	20
P. aquosus T 29/G	12	8

encountered, or only in small degree, with various other *Proactinomyces* strains renders it almost impossible, without further cultural study, to formulate even a tentative hypothesis in explanation.

DISCUSSION

With the accumulation of data on the process of steroid degradation by the microflora of soils, the fundamental importance of these organisms in the economy of nature becomes apparent. The mechanism of the synthesis of the polyhydro*cyclo*pentenophenanthrene compounds, both in the animal and vegetable kingdoms, is still obscure, and the complex processes of metabolism, resulting in the biogenesis of the sex hormones and other similar products, has still to be finally elucidated. These processes of synthesis, however, and the subsequent bacterial disposal of the steroid materials when divorced from the living organism may be regarded as constituting a natural 'steroid cycle'. Decomposition of steroids deposited in soils is, in general, a relatively rapid process, although in adverse environmental conditions bacterial activity is restricted and some steroid accumulation results.

In the oxidation of sterols with the 17 side chain intact *Proactinomyces* still appears to occupy a unique position, but a detailed investigation (Turfitt, 1947) of the organisms utilizing other steroids where the side chain is not present indicates that various Gram – organisms may also play a not inconsiderable role in the disposal of this type of compound, whilst moulds and actinomycetes are more or less inactive in this respect.

The products of steroid breakdown do not, so far as they have been individually isolated and investigated, appear to have any significance as plant growth stimulants. The possibility of phytohormone activity in the further disruptive products has been examined experimentally in the case of the germination of cress seeds, and also in the root formation and growth promotion of lettuce and tomato seedlings. Two methods were investigated in this connexion; in the first method, cholesterol, carefully purified via the dibromide, was intimately mixed with the experimental soil, and the seeds or seedlings watered daily with a suspension of *P. erythropolis*. In the second technique, no steroid was initially added to the soil, but the latter was treated with a heavy aqueous suspension of *P. erythropolis*; subsequently, watering was carried out with aqueous solutions or suspensions of the crude isolates from the continuous culture method. Comparison with the corresponding controls, however, revealed no indication of stimulant action. In the absence of any contrary evidence, therefore, it must provisionally be considered that bacterial decomposition of steroids is essentially a process for the disposal of waste products.

SUMMARY

1. Further *Proactinomyces* oxidation of steroid ketones, obtained as primary oxidation products of hydroxy steroids, results in disruption of the molecules.

2. Yields of degradation products are small, due largely to their further bacterial decomposition. By a continuous culture process sufficient individual products may generally be isolated to permit of identification, but the bacterial oxidation is in no way comparable with chemical technique as a preparative method.

3. Two types of oxidation are encountered, and are frequently simultaneous processes, viz. ring fission at $>$CO group, and complete removal of side chain giving the *aetio*carboxylic acid.

4. Thus, cholest-4-en-3-one yields 3-keto*aetio*chol-4-enic acid and *iso*caproic acid and Windaus's keto acid by disruption of ring *A* at C_3–C_4.

5. Blocking of free hydroxyl at C_3, as in cholesterol acetate, apparently prevents bacterial fission of the ring at this point, but *iso*caproic acid is again obtained from degradation of the side chain. The expected complementary product, the acetate of 3-hydroxy*aetio*chol-5-enic acid, has not been isolated.

6. Side-chain fission of 3-ketochol-4-enic acid yields 3-keto*aetio*chol-4-enic acid, but no definite product has been isolated as evidence of fission at the 3-keto group.

7. The side chain is also completely removed from 3:7:12-triketocholanic acid giving 3:7:12-triketo-*aetio*cholanic acid, but again there is no conclusive evidence of ring fission at any of the susceptible points.

8. In α-oestradiol the benzenoid ring appears to have an inhibiting influence on the bacterial dehydrogenase system, and it is only when the bacterial population is abnormally high that some slight oxidation to oestrone occurs. No evidence of actual molecular degradation has been obtained.

9. Review of available information suggests the presence of a natural 'steroid cycle', degradation of steroids by soil microflora apparently providing a disposal mechanism for waste materials.

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The Mechanism of the Effect of X-rays on Bacterial Toxins

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PART 1. THE SENSITIVITY OF TOXINS TO X-RAYS

Relatively little work has so far been done on the effect of X-rays on bacterial toxins. Gerhartz (1909) studied the effect of X-rays on diphtheria toxin, and Fabre & Ostrovsky (1912) found a partial inactivation of this toxin following the addition of radium to the toxin solution. Ferroux & Muttermulch (1925) detoxified tetanus toxin with radium and observed that the loss of toxicity was accompanied by a loss of antigenicity. Baker (1935) showed that the sensitivity of tetanus toxin to radium was similar to that of Rous sarcoma virus and bacteriophage, and was less marked than the sensitivity of bacteria. Carpenter & Stokinger (1942), working with culture filtrates of *Clostridium botulinum*, *Cl. perfringens*, *Cl. tetani* and *Staphylococcus aureus*, showed that X-rays caused a partial inactivation of each toxin, tetanus toxin being the most resistant. Loiseleur & Crovisier (1943) were able to increase the effect of X-rays on tetanus toxin by bubbling oxygen through the solution during irradiation.

Most of these investigators, however, were able to obtain only a partial destruction of toxins, and the very wide range and unknown degree of purification of the preparations used in all these works make it quite impossible to draw any conclusions as to the actual sensitivity to X-rays and radium of the toxins used. Moreover, the quantitative results of different experiments cannot be compared and cannot be used as a reliable basis for further investigations. We therefore decided to investigate the conditions favourable for the complete destruction of bacterial toxins by X-rays, the properties of the toxins after irradiation, and the influence of the medium on the sensitivity of toxins to X-rays.

For this purpose two toxins were used, tetanus toxin and *Staph. aureus* haemolysin.

EXPERIMENTAL

Methods

Media used for the production of toxins, and purification of the toxins. Two preparations of tetanus toxin were used.

(1) Crude tetanus toxin. *Cl. tetani* was grown according to Mueller & Miller (1942), and 3% tomato juice was added to the medium. The toxin solution obtained contained 4·5 mg. N/ml., and upon assay on mice was found to contain 1000 minimum lethal doses (Min.L.D.)/ml.

(2) Purified tetanus toxin. This toxin was obtained and purified according to Eaton & Cronau (1938). The purified solution contained 0·14 mg. N and 1000 Min.L.D./ml.

(3) *Staph. aureus* haemolysin. *Staph. aureus* was grown on the medium of Favorite & Haman (1941). The filtrate contained 0·95 mg. N/ml., and up to a dilution of 1 : 200 was capable of haemolyzing rabbit corpuscles. No purification of the haemolysin was attempted. The haemolysin was assayed as follows: 0·5 ml. of serial dilutions of the

toxin were added to 0·5 ml. samples of a 2% suspension of rabbit red-blood corpuscles in saline, and were incubated for 2 hr. at 37°, after which they were refrigerated overnight. The coagulase activity of the same filtrate was tested according to the method of Williams & Harper (1946).

Irradiation. This was carried out with an Ott tube, operated at a tension of 35 kV., and at a current of 5 ma., with a copper anticathode. The distance between the vessel in which the toxin was irradiated and the focal point was 50 mm., and the intensity of the X-rays was about 90,000 r./min. The window consisted of aluminium foil 30 μ. thick.

In the case of tetanus toxin, 0·1 ml. samples of toxin solution containing 100 Min.L.D. were irradiated in cups 15 mm. in diameter. No visible changes were induced in the solutions by irradiation. A control sample (0·1 ml.) containing 100 Min.L.D., kept near the experimental sample during the irradiation, was serially diluted with saline so that 0·5 ml. of the final solution contained 1–50 Min.L.D. After irradiation, the treated sample was diluted serially in an identical manner. At least four white mice, averaging 20 g. in weight, were inoculated intramuscularly with 0·5 ml. of each dilution of the irradiated and the untreated control toxin.

Irradiation of haemolysin was done under the same conditions as for tetanus toxin; 0·1 ml. was irradiated in the same type of cup, and the assays were performed by using identical serial dilutions of irradiated and control samples.

RESULTS

Tetanus toxin. Purified tetanus toxin is destroyed by irradiation much more readily than crude toxin. Comparison of Tables 1 and 2 shows that a dose of 7 million r., under the conditions of the experiment, completely destroyed the purified toxin, whereas the same dose reduced the activity of the crude toxin preparation by only 20%.

X-irradiation destroys the toxicity and the antigenicity of tetanus toxin simultaneously. An attempt was made to immunize mice with purified irradiated tetanus toxin. Purified tetanus toxin was irradiated with 7 million r., thereby completely destroying its toxicity. Twelve mice were intramuscularly inoculated with this irradiated toxin, each mouse receiving four injections, at 2-day intervals, of a dose corresponding to 100 Min.L.D. of the original potency. Three weeks after the last injection the mice were divided into three groups, receiving respectively 2, 5 and 10 Min.L.D. of the original unirradiated toxin. A control group of 12 untreated mice received the same doses at the same time.

All the 24 mice both in the control and the experimental groups died, those receiving 10 Min.L.D. after 3 days, those receiving 5 Min.L.D. after 5 days and those receiving 2 Min.L.D. after 6 days, irrespective of whether they had previously received irradiated toxin or not. This clearly demonstrates that X-ray irradiation destroys the antigenicity of tetanus toxin in the same dose that destroys its toxicity.

Staphylococcus haemolysin. Table 3 shows that, under the conditions of the experiment, 5 million r. completely destroyed the activity of our haemolysin

Table 1. *Irradiation of purified tetanus toxin*

X-ray doses in millions r.

	7			5				4				3				2			Control			
Min.L.D.	100	50	20	100	50	20	10	100	50	20	10	50	20	10	5	20	10	5	20	10	5	1
Days																						
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	T.	T.	.
2	.	.	.	T.	.	.	.	T.	.	.	.	T.	T.	.	.	T.	T.	.	.	+	.	T.
3	.	.	.	+	.	.	.	+	.	.	.	+	T.	.	.	+	.	.	.	.	+	.
4	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	+	.	.	.	.	+
5–10	0	0	0	.	0	0	0	.	0	0	0	.	Δ	.	0	.	.	0	.	.	.	.

+ death; T. tetanus; Δ recovery; 0 unaffected.

Table 2. *Irradiation of tetanus toxin prepared in a synthetic, protein-free medium*

X-ray doses in millions r.

	7				5					4					3				2			Control			
Min. L.D.	100	50	20	10	100	50	20	10	5	100	50	20	10	5	50	20	10	5	20	10	5	20	10	5	1
Days																									
1	.	T.	.	0	.	.	T.	0	0	.	.	T.	0	0	.	T.	.	0	.	T.	.	.	T.	0	0
2	.	+	T.	.	.	.	.	.	0	.	.	.	T.	.	.	+	T.	.	.	.	T.	.	T.	T.	T.
3	.	.	.	.	.	.	+	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	+	T.	.
4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T.	.	.	.	.
5	.	.	T.	0	.	.	.	T.	0	.	.	.	T.	0	.	.	T.	0	.	+	T.	.	.	+	+
6–10	.	.	Δ	.	.	.	.	Δ	.	.	.	.	Δ	.	.	.	Δ	.	.	.	Δ	.	.	.	.

+ death; T. tetanus; Δ recovery; 0 unaffected.

preparation, while smaller doses led to only partial destruction.

Destruction of staphylococcus coagulase by X-rays was tested with the same filtrate that was used as haemolysin. It was found that a dose of 3 million r. completely destroyed the coagulase activity.

Table 3. *Irradiation of staphylococcus haemolysin*

	X-ray doses in millions r.				
Dilutions	5	3	2	1	Control
1/10	0	±	+ +	+ +	+ +
1/20	0	±	+	+ +	+ +
1/50	0	0	±	+	+
1/100	0	0	0	±	+
1/200	0	0	0	0	±

+ + complete haemolysis; + almost complete haemolysis; ± partial haemolysis; 0 unaffected.

X-rays simultaneously destroy the toxicity and the antigenicity of staphylococcus haemolysin. A rabbit was intravenously inoculated every 2 days with 0·3 ml. of irradiated haemolysin, receiving a total of twelve injections. A control rabbit received an identical series of injections of unirradiated active haemolysin. Three weeks after the last injection serum from each rabbit was tested for its haemolysin-neutralizing power. As a control we also tested normal rabbit serum. The sera were serially diluted up to 1:2000; 0·1 ml. of haemolysin was added to 0·1 ml. of each dilution.

Table 4. *Action of immune sera* (*staphylococcus haemolysin*) *on rabbit corpuscles*

Serum dilutions	Serum of animals immunized with irradiated haemolysin	Serum of animals immunized with active haemolysin	Normal serum control
1/10	0	0	0
1/20	0	0	±
1/50	+	0	+
1/100	+ +	0	+ +
1/200	+ +	0	+ +
1/500	+ +	0	+ +
1/1000	+ +	0	+ +
1/2000	+ +	+	+ +

+ + complete haemolysis; + almost complete haemolysis; ± partial haemolysis; 0 unaffected.

Table 4 shows that both normal serum and serum from the rabbit that had been inoculated with irradiated haemolysin prevented haemolysis up to a dilution of 1 : 20, whereas serum from the rabbit which had been inoculated with active, unirradiated haemolysin prevented haemolysis up to a dilution of 1 : 2000. Thus, irradiation of staphylococcus haemolysin destroyed its antigenicity together with its haemolytic power.

PART 2. THE INFLUENCE OF ACCOMPANYING SUBSTANCES IN THE MEDIUM ON THE SENSITIVITY OF BACTERIAL TOXINS TO X-RAYS

The mechanism of the effect of irradiation on toxins and other biological materials was first studied by investigating the effect of the concentration of the biological substance on the ease with which it was destroyed. It was very quickly observed that the substances in the medium accompanying the active biological material were of the greatest importance in determining the sensitivity of the material to irradiation.

As early as 1909, Courmont & Nogier showed that the inactivation of tetanus toxin by ultraviolet rays proceeded much more rapidly if they diluted the medium with distilled water rather than with bouillon. They explained this effect by assuming that the bouillon contained substances which protected the toxin from the effect of ultraviolet radiation. Friedewald & Anderson (1941), working with Shope papilloma virus, showed that the purification of extraneous proteins from the medium greatly decreased the resistance of the virus to X-rays, whereas the addition of protein to the purified virus solution increased its resistance to X-rays. Dale (1940, 1942) and Tytel & Kersten (1941) found that the sensitivity of enzymes to X-ray irradiation was dependent on the degree of purification of the preparation, and at low concentrations on the concentration of the enzyme in the medium. Similar results were obtained by Goldhaber & Leibowitz (1943) with ultraviolet radiations, and by Luria & Exner (1941) in the action of X-rays on bacteriophage. Evans, Slaughter, Little & Failla (1942) carried out detailed experiments on the influence of foreign proteins and a peptide on the X-ray sensitivity of the fertilizing power of *Arbacia* spermatozoa. Lea, Smith, Holmes & Markham (1944) noted the protective effect of gelatin on the inactivation of tobacco mosaic virus by X-rays.

All these studies make it quite clear that proteins, their degradation products and other organic compounds protect a wide range of biologically active materials from the destruction normally caused by electromagnetic radiations. It seemed interesting, therefore, to determine whether the same effect could be demonstrated with bacterial toxins, and whether any light could be thrown on the mechanism of such an effect.

EXPERIMENTAL

Materials used. Proteins: crystalline egg albumin, bovine serum globulin, casein, gelatin, protamine, edestin.

Peptones: albumin, serum globulin, casein peptones.

Protein hydrolysates were prepared from egg albumin, serum globulin and casein by autoclaving for 10 hr. with

25% (v/v) sulphuric acid. The latter was removed as $BaSO_4$, and the filtrate neutralized with NaOH.

Amino-acids: the mixture of amino-acids used was prepared in the proportions in which they are found in egg albumin (Schmidt, 1938). Separate amino-acids were also tested.

Reducing agents: ascorbic acid, thioglycollic acid and glutathione.

Oxidizing agents: cystine and maleic acid.

Procedure. Each of the above substances was added in varying concentrations to the toxin preparations which were then irradiated with a dose sufficient to destroy the toxin in the absence of any additions. Careful controls were set up to ascertain whether the added substance by itself in any way affected the activity of the toxin, as for example by a change of pH, oxidation, etc. After irradiation the toxin was assayed in order to determine the lowest concentration of the added substance that would protect the toxin from X-ray inactivation, under the conditions of the experiment.

RESULTS

The protective effect of proteins

Tables 5 and 6 show that tetanus toxin was protected by egg albumin in a concentration as low as 0·5%, while haemolysin was protected by a concentration of 2·5%. In the case of serum globulin, a concentration of 2·5% was insufficient to protect tetanus toxin, but haemolysin was protected in a concentration of 1% (see Table 6).

Protamine was tested only on tetanus toxin, and was found to protect it in a concentration as low as 0·5%. Casein, gelatin and edestin were tested up to a concentration of 2% on tetanus toxin and on staphylococcus haemolysin, and were found to have no protective effect whatsoever.

The protective effect of peptones

From Tables 6 and 7 it can be seen that albumin peptone protects tetanus toxin in a concentration of 2%, and haemolysin in a concentration of 5%; that globulin peptone completely protects tetanus toxin and haemolysin in a concentration as low as 2%. Casein peptic, tryptic and papain peptones were all found to have no protective effect in a concentration as high as 5%.

Methylation of the albumin peptone used above with diazomethane did not decrease its protective effect, demonstrating that the free carboxyl groups are not essential for this property.

Comparing the protective effects of proteins and peptones, it is clear that the latter are considerably more effective than their corresponding proteins. This is particularly clear in the case of serum globulin, which does not protect tetanus toxin at all up to a concentration of 2·5%, whereas globulin peptone completely protects it at a concentration of 2%.

The protective effect of amino-acids

In order to determine whether the total molecular structure of the proteins and peptones or the amino-acid building blocks were the agents responsible for the effects studied, acid hydrolysates of proteins, various amino-acid mixtures and single amino-acids were tested.

Table 5. *Effect of egg albumin on the inactivation of tetanus toxin by X-rays (irradiated with 7 million r.)*

	Albumin																					Control			
	10%					5%				2%			1%			0·5%			0·1%						
Min. L.D.	50	10	5	2	1	50	10	5	2	50	10	5	50	10	5	50	10	5	50	10	5	50	10	2	1
Days																									
1	+	+	+	T.	T.	+	T.	T.	0	T.	.	.	T.	.	.	0	.	.	0	0	0	+	+	T.	T.
2	.	.	.	+	.	.	+	T.	.	+	T.	0	T.	T.	.	T.	.	.	.	.	.	.	.	+	.
3	.	.	.	.	+	.	.	+	T.	.	+	0	+	T.	0	T.	T.	.	.	.	.	.	.	.	+
4	.	.	.	.	.	.	.	.	T.	.	.	.	.	.	.	+	T.	.	0	0	0	.	.	.	.
5–10	.	.	.	.	.	.	.	.	Δ	.	.	.	.	Δ	.	.	Δ	.	.	.	.	.	.	.	.

+ death; T. tetanus; Δ recovery; 0 unaffected.

Table 6. *Effect of proteins and peptones on the inactivation of haemolysin by X-rays (irradiated with 5 million r.)*

	Albumin (%)			Serum globulin (%)			Albumin peptone (%)					Globulin peptone (%)				Control		
Haemolysin dilution	5	4	2·5	4	2·5	1	5	2	1	0·5	0·1	2	1	0·5	0·1	.	.	.
1/10	++	++	0	++	+	±	++	++	++	+	0	++	++	+	±	.	++	.
1/20	++	+	0	+	+	0	++	++	+	±	0	++	+	+	±	.	++	.
1/50	+	0	0	+	±	0	++	+	0	0	0	+	+	0	0	.	++	.
1/100	+	0	0	±	−	0	+	0	0	0	0	±	0	0	0	.	+	.

Table 7. *Effect of peptones on the inactivation of tetanus toxin by X-rays (irradiated with 7 million r.)*

	Albumin peptone (%)																			
	2				1				0·5				0·1				Control			
Min.L.D.	50	10	5	2	50	10	5	2	50	10	5	2	50	10	5	2	50	10	5	2
Days																				
1	+	T.	T.	0	T.	T.	.	.	.	.	.	.	.	.	.	.	+	+	T.	.
2	.	+	.	.	+	T.	T.	T.	T.	.	.	.	.	.	.	.	.	.	+	T.
3	.	.	+	T.	.	+	.	T.	.	T.	.	.	.	.	.	.	.	.	.	.
4	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	+
5–10	.	.	.	Δ	.	.	Δ	Δ	.	Δ	0	0	0	0	0	0	.	.	.	.
	Globulin peptone (%)																			
1	+	T.	T.	.	T.	.	.	.	.	.	.	.	.	.	.	.	+	+	T.	.
2	.	+	.	.	+	T.	.	.	T.	.	.	.	.	.	.	.	.	.	+	T.
3	.	.	+	T.	.	.	T.	.	.	.	.	.	.	.	.	.	.	.	.	.
4	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	+
5–10	.	.	.	.	.	.	Δ	.	+	0	0	0	0	0	0	0	.	.	.	.

+ death; T. tetanus; Δ recovery; 0 unaffected.

Acid hydrolysates of egg albumin, serum globulin and casein were studied and were found to be much less effective than the original proteins and peptones. Thus, albumin and globulin hydrolysates showed partial protection of both toxins at 5 %, very little at 2 %, and none at all at 1 %. Casein hydrolysate did not protect at all, up to 5 %. An amino-acid mixture, in the proportions present in egg albumin, was approximately as effective as were the acid hydrolysates, and no more. It should be noted that the mixture contained cystine, and not cysteine.

Of all the amino-acids tested singly, only tryptophan, cysteine and methionine were effective as protective agents, while all the others, singly or in groups, were ineffective. Thus, almost complete protection of haemolysin was obtained with cysteine at 0·1 %, with tryptophan at 0·5 % and with methionine at 2 %. Tyrosine could not be tested because at pH 7 it was insoluble in adequate concentrations. Glutathione was an effective protective agent in a concentration of 0·5 %. Cystine on the other hand, was totally ineffective.

At this point it became obvious that the effective amino-acids were those that had reducing properties, and additional reducing agents were therefore tested.

Reducing and oxidizing substances

The results with these substances are summarized in Tables 8, 9 and 10. Thioglycollic acid was found to afford protection of haemolysin in a concentration of 0·5 % and of tetanus toxin in a concentration of

Table 8. *Effect of reducing substances upon the inactivation of haemolysin by X-rays (irradiated with 5 million r.)*

	Cysteine (%)			Cystine* (%)		Glutathione (%)			Thioglycollic acid (%)			Methionine (%)			Tryptophan (%)			Control		
Haemolysin dilutions	1	0·1	0·05	0·5	0·1	0·5	0·1	0·05	0·5	0·1	0·05	2	0·5	0·1	1	0·5	0·1	.	.	.
1/10	++	++	+	±	−	++	+	−	++	++	±	++	++	±	++	++	+	.	++	.
1/20	++	+	±	±	−	±	±	−	++	+	±	+	+	−	+	+	±	.	++	.
1/50	+	+	−	−	−	−	−	−	±	±	−	±	−	−	±	±	−	.	+	.
1/100	±	−	−	−	−	−	−	−	±	−	−	−	−	−	−	−	−	.	+	.

* Present in the form of a precipitate.

Table 9. *Effect of reducing substances on the inactivation of tetanus toxin by X-rays (irradiated with 7 million r.)*

	Cysteine (1 %)				Cysteine (0·5 %)				Thioglycollic acid (1 %)				Thioglycollic acid (0·5 %)				Control			
Min. L.D.	50	10	5	2	50	10	5	2	50	10	5	2	50	10	5	2	50	10	5	2
Days																				
1	+	.	.	.	T.	.	.	.	+	.	.	.	T.	.	.	.	+	+	.	.
2	.	T.	.	.	.	.	.	.	.	T.	.	.	+	.	.	.	.	.	T.	.
3	.	.	T.	.	+	T.	.	.	.	.	T.	.	.	T.	.	.	.	.	.	T.
4	.	+	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	+	.
5–10	.	.	Δ	0		Δ	0	0	.	.	Δ	0	.	Δ	0	0	.	.	.	+

Table 10. *Effect of ascorbic acid on the inactivation of haemolysin by X-rays (irradiated with 5 million r.)*

Haemolysin dilutions	Ascorbic acid (%) 0·25	0·05	0·01	0·005	Control
1/20	+ +	+	+	±	+ +
1/50	+	+	±	−	+
1/100	±	±	−	−	±

1%. Since a number of the effective substances contained SH groups an attempt was made to demonstrate a protective effect with H_2S. This was not possible, however, since H_2S always destroyed the toxin.

As an example of an entirely different type of reducing agent, ascorbic acid was tested and found to protect haemolysin in a concentration of 0·05%. Thus, ascorbic acid was by far the most effective protective agent of all the substances tested. It should be noted that tetanus toxin was destroyed by ascorbic acid, and it could not therefore be tested on this toxin. All attempts to reactivate the X-ray-destroyed haemolysin with ascorbic acid were entirely unsuccessful, thus suggesting that the effect of X-rays on haemolysin cannot be reversed by reduction.

Oxidizing substances such as cystine and maleic acid were totally ineffective as protective agents.

DISCUSSION

The experiments reported above in Part 1 provide an additional example of a phenomenon repeatedly observed with biological materials, namely, that as long as the substance studied is not absolutely pure, it is impossible to draw any conclusions as to its specific sensitivity to X-rays. Thus, partially purified tetanus toxin was found to be far more sensitive to X-irradiation than a crude preparation, 7 million r. destroying 100% of the purified toxin, but only 20% of the crude toxin. Moreover it seems probable that further purification would have increased the sensitivity of the toxin even more. These results are very similar to those of Friedewald & Anderson (1941) working with Shope papilloma virus, of Dale (1940, 1942) and Tytel & Kersten (1941) working with various enzymes and of Luria & Exner (1941) working with bacteriophage, etc.

Comparing the toxins we have studied, it is probable that the haemolysin is more sensitive to X-rays than the tetanus toxin, since the purified tetanus toxin solution containing 0·14 mg. N/ml. was approximately twice as resistant as the crude haemolysin solution containing 0·95 mg. N/ml.

In the case of both of these two toxins, the antigenicity was found to be destroyed simultaneously with the toxicity, thus precluding the use of X-rays for the production of toxoids. This phenomenon is not a general one, as it was found by various authors that toxicity and infectivity were sometimes more easily destroyed by X-rays than were antigenic properties (Friedewald & Anderson, 1941; Moore & Kersten, 1936).

Turning to the mechanism of the action of X-rays on toxins (Part 2), it is now generally accepted that the effect of X-rays on substances in dilute aqueous solution is indirect, the radiation causing ionization and other dissociations of water molecules, which produce OH^- and H^+ ions, H_2O_2 molecules, H and HO_2 radicals and other active groups. The end result will depend on whether the substance in solution is more easily destroyed by oxidation or by reduction (Fricke, 1935; Weiss, 1944).

In the case of simple organic substances it is possible to tell by analysis whether the effect of X-rays has been one of oxidation or reduction. In the case of complex, biologically active materials this is obviously impossible, and it is therefore necessary to resort to the study of the protective effects of added extraneous substances.

In the case of the toxins here employed the conclusion that X-ray destruction in dilute aqueous solution is brought about by oxidation of the toxin fits all the observed facts well. Indeed, the ability of a substance or a mixture to protect the toxin from destruction by X-rays was in direct proportion to the magnitude of its reducing properties.

(1) The only amino-acids that were effective, cysteine, methionine, and tryptophan, were those known to have reducing properties or that could acquire them on irradiation (Mirsky & Anson, 1936). Glutathione evidently acted in the same way.

(2) Reducing substances entirely unassociated with proteins, such as thioglycollic acid and ascorbic acid, were among the most effective protective agents.

(3) Oxidizing substances, such as cystine and maleic acid, had absolutely no effect.

It is very interesting to note that Forssberg (1947), working with solutions of pure catalase, has observed the opposite effect, namely, that the destructive effect of X-rays on catalase in dilute aqueous solution is prevented by oxidizing substances, such as cystine, maleic acid, oxidized glutathione, etc., and enhanced by reducing agents such as cysteine, glutathione, etc. The obvious conclusion is that catalase was sensitive to reduction whereas the toxins used in this study were sensitive to oxidation.

On this basis, it is possible to explain a number of observations that would otherwise be very obscure. The peptones were found to be more effective than the corresponding proteins, since reducing groups are presumably set free during partial hydrolysis. The total acid hydrolysates of the proteins were less effective than the proteins themselves, since tyrosine and tryptophan are destroyed and cysteine may be partially oxidized during the process.

It is possible to explain the mechanism of the protective effect of proteins in one of several ways: (1) The protein molecules in solution may act by screening the substance in solution from the destructive effect of X-rays, i.e. passive protection; (2) active protection may be induced, whereby the protein molecules actually intervene in the reaction between the active groups produced in the water and the substance in solution, either by combining with the substance and thus stabilizing it (Tytel & Kersten, 1941) or by reacting with the active groups thus inactivating them.

The experiments reported above clearly show that the protective effect of the proteins cannot be explained by the first mechanism, since it is not dependent on the molecular size and shape or on the amount of extraneous materials in the solution. Indeed, a number of proteins, such as casein, gelatin and edestin, and many amino-acids singly or in combination are completely inactive even in very high concentrations, although other proteins, such as egg albumin and serum globulin, and some amino-acids very similar in their general molecular structure to the inactive ones, are very active in low concentrations. It is also extremely improbable that the same toxin can combine with such a wide variety of molecules as were found to be effective protective agents, thus excluding the explanation that the protective agents react with the toxin and stabilize it.

We are thus left with the last possibility, namely, that the protective agents combine with the active groups formed in the water and destroy their activity. The native proteins here tested show no reducing properties, but it is known that denaturation sets free reducing groups, e.g. SH groups (Mirsky & Anson, 1936) and, since X-rays cause denaturation of proteins, it is reasonable to believe that the protective effect of proteins was due to the reducing power of their denatured products, just as in the case of the reducing amino-acids.

Gelatin has only very slight reducing properties, and it was found to have no protective effect on the toxins tested. It is noteworthy that gelatin acts as a very effective protective agent for tobacco mosaic virus against X-rays (Lea *et al.* 1944). The mechanism in that case must be different from the one which is effective for tetanus toxin and staphylococcus haemolysin.

SUMMARY

1. Tetanus toxin and staphylococcus haemolysin were destroyed by X-rays. Purification of tetanus toxin greatly increased its sensitivity to X-rays.

2. In both cases the antigenicity was destroyed simultaneously with the toxicity.

3. Certain proteins, their partial and total hydrolysis products, reducing amino-acids, glutathione, thioglycollic and ascorbic acids were found to be effective as protective agents, whereas oxidizing substances were ineffective.

4. All the experimental observations support the theory that the mechanism of the effect of X-rays on tetanus toxin and staphylococcus haemolysin is an indirect one, the radiation causing the formation of active oxidizing radicals in the water, which in turn destroy the toxin by oxidation.

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The Purification of Antitoxic Plasmas by Enzyme Treatment and Heat Denaturation

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The work of Parfentjev (1936) refocused attention on the possibility of refining antitoxins contained in immune plasmas by the use of enzymes (originally claimed in British Patent No. 18340, Imray, 1902). Later work by Pope (1938), confirmed by the ultracentrifuge studies of Peterman & Pappenheimer (1941), showed that the molecule of diphtheria antitoxic protein may be split by treatment with certain enzyme preparations into two portions of approximately equal size but having different properties, one fragment containing the whole of the antitoxic property of the original molecule. These smaller protein units could still be heat denatured, and Pope (1939*a*) showed that the rate of denaturation of the non-antitoxic portion was very much greater than that of the portion which carried the antitoxic properties of the original unmodified antitoxic molecules. Pope used these findings as the basis of a method for the purification of antitoxic plasmas, consisting essentially of two stages: (1) The antitoxic plasma is treated with pepsin; this splits the antitoxic molecule into two portions, only one of which bears the antitoxic property of the original. (2) This enzymic splitting is followed by critical heat denaturation in the presence of salts and chemical denaturants to precipitate non-antitoxic protein, leaving the modified antitoxic protein in solution.

Pope's original work was carried out on diphtheria and other antitoxin solutions (Pope, 1938) and the results obtained gave information on which a large-scale process could be based. There was, however, little detailed quantitative knowledge relating to the numerous factors which might influence the yield and the degree of purification of the antitoxic protein.

The object of this paper is to describe the large-scale process eventually evolved for refining horse-blood plasmas containing antitoxins and for the concentration and preparation of the refined products for therapeutic use. The process has an intrinsic interest since it involves reactions with labile biological materials of fundamental importance. The development from the original observations which formed the basis of the method to the large-scale process gave rise to numerous problems not encountered in the laboratory. Further problems arose from the increase in the scale of operation which necessitated different ways of handling (involving heating and filtration) during processing. Since some of the problems encountered may arise from fundamental properties of the plasma constituents they should be recorded, as they may suggest new fields for experimental study. Furthermore, the increase in size of the operations magnifies the effect of some plasma constituents which might otherwise be unobserved; processes in which blood plasma is routinely handled in volumes measured in hundreds of litres are not common.

EXPERIMENTAL

Methods and materials

In outline, the purification process comprises two stages: (*a*) pepsin treatment; (*b*) critical denaturation of non-antitoxic protein on samples of antitoxin solution (obtained by the fractional precipitation of antitoxic plasma with $(NH_4)_2SO_4$, followed by dialysis to remove the $(NH_4)_2SO_4$) or antitoxic plasma, varying one or more of the different factors involved in separate experiments. Thus, the method used in each experiment, unless otherwise specified, was as follows:

(*a*) *Pepsin treatment.* Antitoxin solution or antitoxic plasma was diluted with the requisite volume of cold tap water, the dilution adjusted to the required pH with solid citric acid and pepsin powder added. After the action of the pepsin a sample was taken (stage A), the sample being neutralized to *c.* pH 7 with 40% (w/v) NaOH, filtered and the filtrate analyzed for total protein and antitoxin unitage.

(*b*) *Denaturation.* After the action of the pepsin the required quantity of solid $(NH_4)_2SO_4$ was added and dissolved, the pH adjusted as required with 40% (w/v) NaOH or solid citric acid. Portions of 100 ml. were then heated in a water bath at 55° (±0·5°) in 120 ml. rubber-stoppered glass bottles. The duration of this heat denaturation was measured from the time of immersion in the bath. Samples were taken after the addition of the $(NH_4)_2SO_4$ and adjustment to the pH required for heat denaturation (stage B), filtered and the total protein and antitoxin estimated in the filtrates. Finally samples were taken after heat denaturation (stage C) and similarly analyzed.

Total protein. This was estimated by a refractometric method (Pope, 1939*b*).

Antitoxin. Diphtheria antitoxin has been used except where otherwise stated. The antitoxic content was estimated by the flocculation method (accurate to ±5%). From time to time these results have been checked by intracutaneous injection in guinea pigs (Römer & Sames, 1909).

Degree of purification of antitoxin protein. This was evaluated in terms of antitoxic units/g. protein (u./g.); the increase in purity is denoted by *R*, which represents the ratio of the final u./g. to the initial u./g. (Pope, 1939*b*).

Percentage yields. These have been corrected for the dilution involved when solid $(NH_4)_2SO_4$ is added to a given volume of liquid.

Pepsin. Throughout the work pepsin refers to a powder preparation of B.P. 1 : 2500 quality (Chas. Zimmerman and Co. Ltd., London).

The large-scale process

The process finally evolved, using the data obtained from numerous experiments carried out as just described in order to choose the optimal conditions for each operation, can be divided into the following operations:

(1) Bulking of plasma followed by dilution.
(2) Adjustment of pH and addition of pepsin.
(3) Readjustment of pH and addition of $(NH_4)_2SO_4$.
(4) Heat denaturation and filtration.
(5) Recovery of purified antitoxic protein by precipitation with $(NH_4)_2SO_4$.
(6) Dialysis followed by addition of antiseptic.
(7) Ultrafiltration.
(8) Clarification and adjustment of tonicity.
(9) Sterilization.

Experimental details of the above operations are given below.

(1) *Bulking of plasma followed by dilution.* Bulked plasmas of similar antitoxic value (to a total volume of 140 l.) were used whenever possible as this provided a more constant starting material and gave greater flexibility in the final blending for issue for therapeutic use. Since plasma is viscous it is preferable for handling on a large scale that it should flow freely through pipelines and valves. Experimental study of various dilutions both for pepsin treatment and heat denaturation indicated that a dilution of plasma with 2 vol. of water was optimal (protein concentration 2·5–3·0%) although it was feasible to treat with pepsin at a 1 : 2 dilution and then to add a solution of $(NH_4)_2SO_4$ to give a 1 : 3 dilution for the heat denaturation stage. Dilution was carried out with hot water (55°) to give a final temperature in the mixture of 30°.

(2) *Adjustment of* pH *and addition of pepsin.* Experiment showed that the period usually required for considerable enzymic digestion (24 hr. at 37°) was quite unnecessary for the splitting of the antitoxin molecule to the degree needed to allow the differential heat denaturation. Provided the pH was low, either 2 hr. at 20° or 30 min. at 30° produced sufficient disaggregation to prevent subsequent heat denaturation of the resulting modified antitoxic protein. The pH optimum was 3·2; higher pH values produced insufficient disaggregation over a short period, while lower pH values caused destruction of antitoxin. Although in experimental work citric acid was used, to avoid volume changes, it caused unnecessary buffering of the solution and on a large scale HCl, which could be added either after dilution (as 1 : 4 dilution of conc. HCl), or more conveniently during dilution (as 1 : 15 dilution of conc. HCl), was found preferable. The quantity of pepsin required was small as shown in Table 1; 5 g./l. of plasma were sufficient.

Table 1. *The effect of varying the concentration of pepsin*

(Antitoxic plasma containing 0·70% (v/v) 'tricresol'* + ether mixture (i.e. 0·35% 'tricresol') was diluted 1 : 3. Samples taken after 1, 2 and 3 hr. of pepsin treatment at 19° and pH 3·2, were heated for 2 hr. at pH 4·1 in the presence of ammonium sulphate (140 g./l.). Original material (diluted): protein 2·57%; antitoxin 164 u./ml., 6400 u./g.)

Duration of pepsin treatment (hr.)	Pepsin added (g./l. plasma)	Protein present after denaturation (%)	*R* value	Yield of antitoxin (%)
1	1	0·230	4·21	41·1
	2	0·281	4·56	54·3
	5	0·339	4·23	60·9
	10	0·385	4·01	64·9
2	1	0·254	4·29	46·4
	2	0·332	4·14	58·3
	5	0·411	4·03	70·3
	10	0·403	3·95	67·6
3	1	0·352	4·19	62·3
	2	0·405	3·78	64·9
	5	0·442	3·75	70·2
	10	0·433	3·82	70·2

* 'Tricresol' (Schering) is a liquid mixture of the three isomeric cresols, and is usually added to plasma mixed with an equal volume of diethyl ether, to give 0·35% of 'tricresol' in the plasma.

(3) *Readjustment of* pH *and addition of* $(NH_4)_2SO_4$. The quantity of protein rendered insoluble by heat denaturation and therefore the degree of purification were found to depend on both the pH and concentration of $(NH_4)_2SO_4$ (see Figs. 1, 2). The quantity of antitoxin denatured was also dependent on these two factors as shown by the curves for '% yield'. The maximum yield is of primary importance on a large scale (provided an *R* value of at least 3·0 can be obtained), and for this the essential requirements are high pH and low $(NH_4)_2SO_4$ concentration. It was found, however, impossible to obtain efficient filtration if the pH were above 4·3 and the $(NH_4)_2SO_4$ concentration below 130 g./l.; these values were therefore chosen. (The use of salts other than $(NH_4)_2SO_4$ has not given any improvement and has invariably led to slower rate of filtration. Various adsorbents of the kieselguhr type and also activated charcoal have been added during heat denaturation, but in no case has an increase in purity of the antitoxin been observed, nor have filtration rates been substantially improved.)

At this stage of the process 0·1% toluene is added, which later removes lipid material by adsorption on the heat denatured protein, thus preventing its appearance as a precipitate in the final product. Sodium pyrophosphate (0·2%) is also added to raise the pH and to inhibit the formation of coloured compounds from the 'tricresol', previously added (0·35%) to the plasma as a preservative. The first batches of material processed in stainless steel tanks developed a marked red colour due, as was known from earlier work (Pope, unpublished), to the presence of iron which catalyzed the production of quinones from the 'tricresol'; these react with the proteins to give coloured products. This catalytic action of iron can be inhibited by pyrophosphate (Pope, personal communication), and in all subsequent batches sodium pyrophosphate has been added.

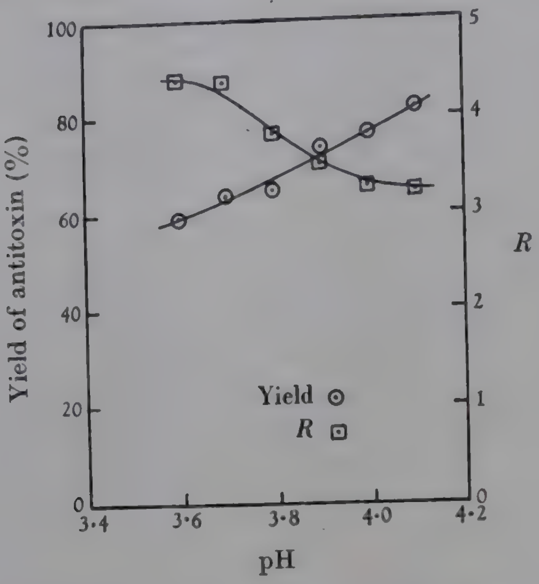

Fig. 1. The effect of pH on the % yield of antitoxin and the degree of purification (R). Antitoxic plasma, diluted 1 : 3 and treated with pepsin powder (5 g./l.) for 2 hr. at pH 3·2 and 18°; heated for 2 hr. in the presence of ammonium sulphate (100 g./l.). Original material (diluted): protein 2·32%; antitoxin 156 u./ml.; 6720 u./g.

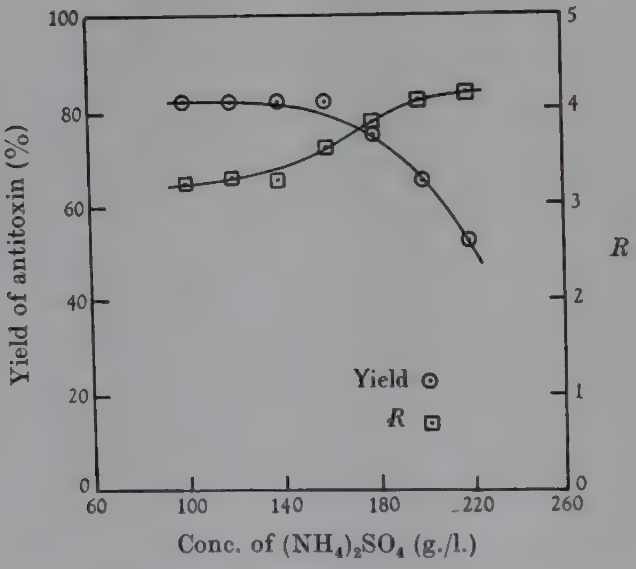

Fig. 2. The effect of ammonium sulphate concentration (g./l.) on the % yield of antitoxin and the degree of purification (R) when antitoxic plasma is treated as in Fig. 1, but heating was at pH 4·1.

(4) *Heat denaturation and filtration.* The mixture prepared as just described and adjusted to pH 4·3, is heated in spray-heated stainless steel vessels for 1 hr. at 55°, during which approximately 65% of the original protein is rendered insoluble. It is important that the temperature should be raised to 55° as quickly as possible (20 min.) otherwise peptic action continues, causing the heat-denatured precipitate to become difficult to filter. Heating may be prolonged, leading to increased purity with further slight loss of antitoxin, but higher temperatures, above 60°, markedly increase the rate of destruction of antitoxin.

The concentration of 'tricresol' present during heat denaturation has a marked effect on the purity as shown in Table 2. The concentration of 'tricresol' usually used in these laboratories as a preservative and which gives a concentration of 0·12% on dilution of the plasma 1 : 3 as above is therefore suitable for obtaining a high yield and degree of purification. Many other compounds such as phenol, chloroform, ethanol, acetone and urea were tested in the denaturation stage but none was found superior to 'tricresol'.

Table 2. *The effect of various concentrations of 'tricresol' on the yield and degree of purification of antitoxin*

(Antitoxic plasma diluted 1 : 4 was treated with pepsin (5 g./l.) for 2 hr. at 18°, pH 3·2. Ammonium sulphate (100 g./l.) was then added and the pH adjusted to 4·1. Various percentages of 'tricresol' were then added and the solution heated for 2 hr. Original material (diluted): protein 2·32%; antitoxin 156 u./ml., 6720 u./g.)

'Tricresol' (%)	Protein in filtrate (%)	R value	Yield of antitoxin (%)
0·00	0·541	3·24	82·2
0·0125	0·531	3·20	79·4
0·025	0·503	3·38	79·4
0·05	0·442	3·84	79·4
0·10	0·368	4·38	75·2
0·15	0·315	4·83	71·0
0·25	0·252	5·09	59·9

After cooling the liquor to 50° the denatured protein is filtered off in a washing-type ebonite plate and frame filter press and the precipitate washed with $(NH_4)_2SO_4$ solution. By carefully controlling the conditions under which the precipitate is formed it is possible to build up a cake 1 in. thick and still obtain filtration in a reasonable time.

On a large scale an average yield of 65% of the original unitage is obtained in the filtrate plus washings, which are quite clear and colourless.

(5) *Recovery of purified antitoxic protein by precipitation with* $(NH_4)_2SO_4$. The purified antitoxic protein is precipitated from the filtrate obtained as described in section (4) by a further addition of between 160–190 g. $(NH_4)_2SO_4$/l., the quantity added being dependent on the type and potency of the original antitoxic plasma. It is important that the amount of $(NH_4)_2SO_4$ added should be as low as possible in order to minimize concurrent precipitation of proteose, formed in the initial stages of the process by peptic digestion of the serum albumin.

This precipitate of modified antitoxic protein is very difficult to filter and a filter press can only be used with a gravity feed having a head of less than 6 ft. It is then possible to build up a cake 0·5 in. thick. For efficient filtration, the precipitation should be carried out while the filtrate is still warm, but if it is allowed to cool the suspension formed by the addition of $(NH_4)_2SO_4$ may be carefully reheated to 50° before filtering. It is usual to allow this filtration to proceed slowly overnight.

If the filtrate is fractionated by the addition of successive portions of $(NH_4)_2SO_4$ it is found that a first fraction, containing about 10% of the total antitoxin, is of lower purity than the remainder. When processing low-grade antitoxic plasma, it is convenient to add an extra 40 g./l. of $(NH_4)_2SO_4$ to the suspension after heat denaturation in order to precipitate this antitoxin. This is then recovered from the filter press by washing and can be worked up as a recovery batch. If other methods for increasing the degree of purification, such as lowering the pH or increasing the $(NH_4)_2SO_4$ concentration for heat denaturation are adopted the additional lost antitoxin cannot be recovered.

(6) *Dialysis followed by addition of antiseptic.* The cakes of antitoxic protein obtained from the filter press contain a large amount of 30% $(NH_4)_2SO_4$ solution. This is removed by dialysis in cellophan sacs against running tap water, until 0·1% $(NH_4)_2SO_4$ remains; this operation takes approximately 48 hr. After bulking the contents of the sacs 0·35% of 'tricresol' is added as a 10% emulsion in water, which is sprayed into the solution through a perforated stirring tube.

(7) *Ultrafiltration.* It was customary in the early days of this process to press the filter-press cakes of antitoxic protein between sheets of blotting paper in order to remove excess $(NH_4)_2SO_4$ solution before dialysis. It was found however that precipitates of denatured protein sometimes appeared in the final product after candling, possibly due to excess pressure during the pressing. When pressing was discontinued the solution of antitoxic protein obtained after dialysis contained only 9% protein, whereas for therapeutic purposes it is desirable to have the solutions as concentrated as possible, without undue viscosity.

With purified and modified antitoxic protein, 15% solutions are not unduly viscous. The method for raising the concentration to about 15% is ultrafiltration, using collodion membranes deposited on the external surfaces of filter candles. These are immersed in a solution of 8% collodion (Imperial Chemical Industries product) in glacial acetic acid and, after draining, gelled in water. The ultrafiltration takes approximately 7–10 days, but has the merit of removing a portion of the proteose present and also of allowing 'tricresol' to come into equilibrium with the protein. It is carried out by running serum into the stainless steel vessel, which contains five ultrafilters, and applying a vacuum of 29 in. to the bottles connected to the ultrafilters (Fig. 3). Concentrated serum is drawn off from the bottom of the stainless steel vessel every 24 hr.

(8) *Clarification.* It is essential that the ultrafiltered solution should be perfectly clarified to ensure easy filtration through filter candles. This clarification is accomplished by drawing the protein solution through a thick pad of lightly packed paper pulp by means of a low vacuum (5 in. Hg). Autoclaved and finely shredded paper pulp is formed into a pad about 1·5 in. thick in a stainless steel vessel shown in Fig. 4.

(9) *Sterilization.* After clarification, the antitoxin solution is made isotonic by the addition of sterile 20·0% (w/v) NaCl containing 0·1% $NaHCO_3$, in order to bring the solution to pH 6·0, which is the optimum for storage and therapeutic use. The antitoxin solution is then drawn through a filter candle (see Fig. 5) using a vacuum not greater than 20 in. Hg in order to prevent frothing, which has a tendency to cause denaturation of protein. Under these conditions for clarification and sterilization, it is possible to pass 20–30 l. of antitoxin solution (corresponding to two batches) through each pulp and candle.

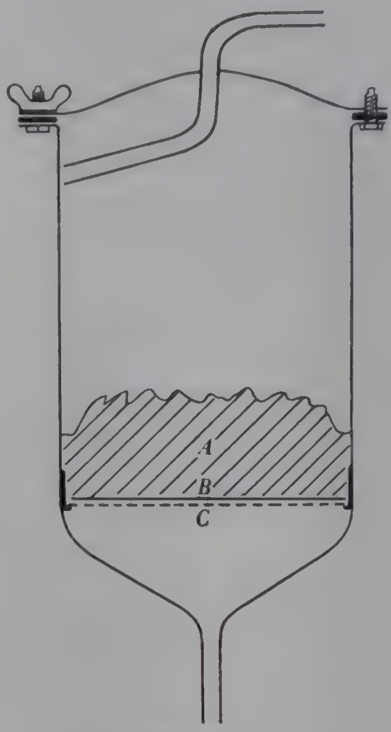

Fig. 4.

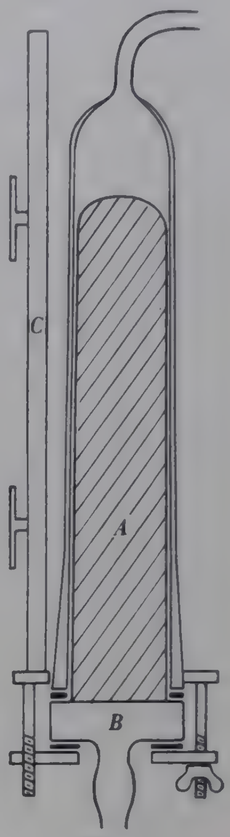

Fig. 5.

Fig. 4. Stainless steel vessel (6 in. diameter) for containing the pad of paper pulp. (*A*) Paper pulp. (*B*) Disk of stainless steel gauze. (*C*) Perforated stainless steel disk.

Fig. 5. Filter candle (10 in.) clamped into glass case (constructed from 2 in. Pyrex pipeline) by means of two stainless steel disks. (*A*) Filter candle. (*B*) Porcelain head of candle. (*C*) Stainless steel supporting bracket.

Applications of the process

Antitoxins. It has been found possible to refine by the process described all true antitoxins, in which the antibody lies largely in the pseudoglobulin fraction of the plasma. A typical series of experimental results is given in Table 3. The R values in

Table 3. *Results obtained by processing various types of antitoxic plasma*

(Typical batches of plasma were processed by the method described.)

Type	R value	Overall yield of antitoxin (%)
Perfringens antitoxin, type A	3·28	57·4
Septique antitoxin	5·38	57·6
Oedematiens α-antitoxin	4·53	52·5
Tetanus antitoxin	3·84	65·5
Scarlet fever antitoxin	4·97	55·4
Staphylococcus α-antihaemolysin	4·33	66·1
Shiga dysentery neuro-antitoxin	2·99	54·3
Diphtheria antitoxin	3·87	63·2

general vary between 2·5 and 5·0 and are to a large extent dependent on the antitoxic potency of the original plasma, i.e. the higher the antitoxic value of the original plasma, the lower the R value. Thus results from 80 batches of diphtheria antitoxic plasma showed a correlation coefficient between R value and antitoxic potency of $-0{\cdot}777$ which is statistically very significant.

The final product is a perfectly clear straw-coloured liquid, not unduly viscous and showing no signs of precipitation when stored, even at room temperature, over a period of 5 years. It is also remarkably stable, the rate of loss of antitoxic potency at the pH optimum (5·5–6·0) being negligible between 0° and + 5°, approximately 5·0 % per annum at 20° and 10·0 % per annum at 37° (Harms, 1945). An extremely low incidence of serum reactions has been reported when antitoxic sera refined in the way described have been used therapeutically (Hutchinson, 1939). It has also been shown by Glenny & Llewellyn-Jones (1938) to be absorbed more rapidly and eliminated more slowly than antitoxin purified only by fractional precipitation with ammonium sulphate.

Antibacterial antibodies. Although the fractionation of sera containing antibacterial antibodies with ammonium sulphate has shown that these antibodies are present in the euglobulin fraction, some experiments on refining these sera by the pepsin method were carried out. The results of processing plasma containing antibodies prepared by using as antigen suspensions of the following organisms, *Haemophilus pertussis*, *Proteus vulgaris* OX 19, *Neisseria intracellularis*, and *Erysipelothrix rhusiopathiae* (the organism of swine erysipelas), showed that, as expected, only extremely small antibody yields (less than 20 %) could be obtained. Recently a method for concentrating such sera, utilizing the denaturing properties of 'tricresol' has been worked out (Harms, 1946).

The behaviour of separated fractions of antitoxic horse plasma during pepsin treatment and heat denaturation. It has long been customary to concentrate the antitoxic fractions of plasmas by fractional precipitation with ammonium sulphate or other neutral salt, so that the pseudoglobulin fraction, containing the bulk of the antitoxin is partially separated from the fibrin, euglobulin and albumin fractions. Earlier work relating to the process described here was carried out on this pseudoglobulin fraction (Pope, 1939*a*) which was found to behave in a manner similar to whole plasma when processed by the pepsin method. It seemed therefore of interest to investigate the behaviour of the euglobulin and albumin fractions. Very laborious procedure is necessary to obtain electrophoretically homogeneous fractions, but a simple fractionation with ammonium sulphate can give fractions which contain a preponderance of one component. Plasma diluted with an equal volume of water was fractionated by precipitation with ammonium sulphate at the concentration indicated: fraction 1 (euglobulin fraction) 0–220 g./l.; fraction 2 (pseudoglobulin fraction) 220–360 g./l.; fraction 3 (albumin fraction) 360–550 g./l. The three precipitates were then dialyzed.

The solution of each fraction was diluted with tap water to contain 5·0 % protein and then an equal volume of 0·24 % 'tricresol' in tap water was added to give a concentration of 'tricresol' suitable for heat denaturation and a protein concentration equal to that obtaining when plasma is used. After treatment with pepsin for 1 hr. at 18·5°, ammonium sulphate (130 g./l.) was added and the pH adjusted to 4·25 with 40·0 % (w/v) NaOH. Samples (100 ml.) were then heated for 1 hr. at 55°. Protein and antitoxin were estimated on samples taken at stage B (after addition of ammonium sulphate) and stage C (after heat denaturation); total N and non-protein N estimations (Kjeldahl) were carried out on samples at stage A (after pepsin treatment), in order to determine the amount of true digestion which took place. Electrophoretic analyses, for which I am indebted to Mr P. A. Charlwood, were also carried out on the untreated fractions. The results are summarized in Table 4.

Although the fractions into which the plasma had been separated by the ammonium sulphate fractionation were by no means homogeneous, they showed marked differences in composition as determined by the electrophoretic measurements. The results of pepsin treatment followed by heat denaturation also show differences in behaviour. It is clear (Table 4) that the albumin is almost wholly digested while the other proteins show very little digestion. The impure (in terms of u./g. value) fraction 1 gave a degree of purification almost as high as that obtained with fraction 2, due largely to the fact that a very considerable portion of the protein was precipitated by the addition of ammonium sulphate (stage B). It seems logical to suppose that the protein thus precipitated is largely γ-globulin and fibrin (estimated electrophoretically under β-globulin). Most of the antitoxin would appear to be carried by the β-globulin, which apart from being split by enzyme action was largely unaffected by the processing. The figures for fraction 3 however appear to indicate that a part of the β-globulin had no antitoxin associated with it.

The fractions showed marked differences in rate of filtration. Samples from fraction 1 filtered very rapidly and gave brilliantly clear filtrates, whereas with fractions 2 and 3 the filtrates tended to be opalescent. The precipitates formed from fraction 3 were also very sticky and filtered very badly. The good filtration obtained with fraction 1 is in accordance with other observations in which it has been noted that filtration is always better when plasma

Table 4. *The effect of processing fractions of antitoxic horse plasma*

(Antitoxin and protein remaining in solution after processing are given as % of that present in the original fraction.)

			Antitoxin				Electrophoretic composition (%)						Antitoxin				Non-protein N/total N (%)
Fraction	% of total protein	Protein conc. (%)	u./ml.		u./g.		Globulin			Albumin	Stage in process	Protein (%)	(%)		u./g.		
			*In vitro**	*In vivo*†	*In vitro**	*In vivo*†	γ	β	α				*In vitro**	*In vivo*†	*In vitro**	*In vivo*†	
1	33·0	2·52	125	125	4,960	4,960	40·9	38·2	16·3	4·6	A	81·4	—	—	—	—	39·6
											B	36·7	87·0	—	11,600	—	—
											C	23·4	80·8	81·8	17,000	17,200	—
2	47·0	2·55	250	220	9,790	8,620	16·8	43·0	32·9	7·3	A	80·4	—	—	—	—	33·8
											B	70·1	87·1	—	12,000	—	—
											C	37·4	79·7	93·6	20,700	21,400	—
3	20·0	2·54	—	1·5	—	—	0·0	13·5	9·5	77·0	A	31·6‡	—	—	—	—	61·3
											B	25·7	—	—	—	—	—
											C	5·5	—	—	—	—	—

* *In vitro* = measured by the flocculation test.

† *In vivo* = measured by intracutaneous injection into the skin of guinea pigs (Römer & Sames, 1909).

‡ After 2 min. pepsin treatment the protein concentration had diminished to 75·7 % of the original.

is processed rather than serum. There is, therefore, no necessity to convert plasma to serum before processing; in fact it is a disadvantage. It would be an advantage to remove albumin since this would lead to better filtration and obviate the production of proteose and peptone, which tend to contaminate the final product. This, however, would necessitate a laborious initial process involving a difficult filtration.

The treatment of whole blood. An experiment with whole horse blood in comparison with plasma obtained from the same bleeding was carried out. The experiments recorded in Table 5 show that there was a comparatively small increase in total units recovered, while the degree of purification was slightly less when whole blood was used. Concentrates prepared from the filtrates were very much darker in colour; and there would be a danger that breakdown products of haemoglobin might appear in the concentrates. No advantage would therefore appear to be gained by processing whole blood.

Table 5. *The effect of processing whole blood*

(500 ml. of blood were divided into two parts. (A) 250 ml. blood + 750 ml. tap water + 0·12 % 'tricresol', were treated with pepsin (5 g./l.) at pH 3·2 for 2 hr. at 19°. Heat denaturation for 1 hr. at 55°, pH 4·1. (B) 250 ml. of blood were allowed to settle for 24 hr. and the supernatant plasma siphoned off. 184 ml. of plasma were thus obtained and after dilution with 2 vol. of tap water, 0·12 % 'tricresol' was added and the solution treated as (A). The cells and their suspending plasma, after withdrawal of the supernatant plasma, were diluted to 250 ml. with tap water, 0·12 % 'tricresol' was added and the solution was treated as (A). Antitoxin was recovered as recorded below. Original material: blood (diluted): protein 3·15 %, antitoxin 204 u./ml.; 6500 u./g. Plasma (diluted): protein 2·64 %, antitoxin 320 u./ml.; 12,100 u./g. Total antitoxin units in 250 ml. blood = 204,000.)

	Units recovered		
Material	Total	% of original*	u./g.
Blood	89,225	43·7	40,000
Plasma	82,325	40·4	42,500
Cells	5,719†	2·8	45,400

* Low yields due to mechanical loss in processing small quantities.

† Presumably from the plasma left with the cells.

DISCUSSION

The results obtained both in the laboratory and in large-scale processing show that, in general, disaggregation of a major portion of the antitoxic protein molecules, as judged by the resistance of the antitoxic portion to heat denaturation, takes place very rapidly, even in the presence of very small quantities of pepsin. The destruction of antitoxin which may vary from 20 to as much as 50 % in some batches (even under optimal conditions) may be explained partly by protein denaturation and partly by the resistance of a portion of the antitoxic protein to disaggregation.

When antitoxic sera (including those obtained by the process described herein) containing phenolic preservatives are stored for a long period a small loss in antitoxic potency occurs; this loss is greatly accelerated by rise in temperature and by alterations of pH from the optimum (pH 5·5–6·0). This loss is caused by denaturation of the antitoxic protein (Harms, 1945) and it is probable that a similar process operates during pepsin treatment at pH 3·2 to account for the 10 % loss in antitoxic units which invariably occurs at this stage. Further loss, probably due to the same cause, occurs during the heat treatment, since this loss may be increased by raising the temperature or increasing the time of heating. Whereas the loss of antitoxic units during pepsin treatment is constant, that occurring during the succeeding stages (B and C) of the process is very variable and cannot be accounted for by heat denaturation of disaggregated antitoxic protein. Thus, when ammonium sulphate is added at stage B a quantity of antitoxic protein, which varies considerably from batch to batch, is precipitated. Should the quantity of ammonium sulphate added be lowered in order to decrease this precipitation it is found that an extra loss equivalent to this increase occurs during stage C. The most probable explanation of this phenomenon is that the globulin which can be precipitated by a low concentration of ammonium sulphate carries an antitoxic protein resistant to disaggregation by pepsin treatment. This is confirmed by the observation that when antitoxic globulin solutions prepared by the ammonium sulphate fractionation of immune plasma (during which a fraction of the globulin, insoluble in low concentrations of ammonium sulphate, is removed) are processed, yields are generally higher than when immune plasmas are processed.

Rosenheim (1937) found that whereas *Eberthella typhosa O* agglutinins were destroyed by pepsin treatment, *H* agglutinins were more resistant and the resistance depended on the degree of immunization of the horse from which they were obtained. Also it has been found that antibacterial antibodies are almost completely destroyed when processed by the method described in this paper. The work of Cohn, Oncley, Strong, Hughes & Armstrong (1944) on the ethanol fractionation of human plasma indicates that many of the antibodies, including antitoxins, are associated with the γ-globulin fraction. It is possible that the method of immunization of horses affects the distribution of antitoxin between the β-globulin and γ-globulin fractions. If, as evidence would seem to indicate, the γ-globulin of antitoxic horse plasma is more resistant to disaggregation by pepsin than the β-globulin, this may account for the destruction of antibacterial antibodies because these are largely confined to the γ-fraction and it might account for the variable yields obtained with different batches of antitoxin. The experiment (Table 4) in which three fractions of antitoxic horse plasma were processed shows that very much more protein was precipitated and heat-denatured in fraction 1, containing the high proportion of γ-globulin, than in fraction 2 which contained a much smaller proportion. Although the yield of antitoxin from both fractions was substantially the same, this can be explained by the fact that fraction 1 still contained a high percentage of β-globulin, with which the majority of the antitoxin was associated.

Further work on pure globulin fractions is indicated in order to clarify this point, for it has been suggested that the loss of certain portions of the antitoxic protein during purification may have led to the failure of antitoxin therapy in some types of diphtheria infection (O'Meara, 1939).

The purity of antitoxin (u./g.) which can be obtained depends mainly on the purity of the original plasma, although the presence of antitoxins other than the specific one, formed either during the course of immunization or during a previous immunization to produce another type of antitoxin, will contribute protein to the filtrate after heat denaturation and so lower the final purity.

Should it be possible to produce an immunization in a horse in which only one specific antitoxin was formed and the horse possessed no significant quantity of other antitoxin, it seems possible that 'pure' antitoxin could be produced by the pepsin process. Using diphtheria antitoxic plasma containing 3600 units of antitoxin/ml. it was possible to obtain an antitoxin solution of 100,000 u./g., which is of the same order as that produced from refined antitoxin by the method of Pope & Healey (1939) using toxin-antitoxin floccules. Experimental evidence indicates that, even under optimal conditions, the degree of purification and yield of antitoxin which can be obtained by pepsin treatment followed by heat denaturation are limited by the quality of the original plasma. Improvement may therefore come from better methods of immunization designed to produce only the specific antitoxin in a form in which it is easily disaggregated by pepsin.

SUMMARY

1. A description is given of a large scale process suitable for the purification of true antitoxins, but not for antibacterial antibodies, by treatment of immune plasmas with pepsin followed by critical heat denaturation in the presence of a protein denaturant. The preparation of the purified antitoxin for therapeutic use is described.

2. It has been established that the optimal conditions on the large scale are pepsin treatment (0·5 hr. at 30° at pH 3·2 with plasma diluted 1 : 3) and heat denaturation (1 hr. at 55° at pH 4·3 in the presence of 130 g. ammonium sulphate/l. and 0·12 % 'tricresol').

3. It has been shown that it is preferable to process plasma rather than whole blood, serum, or salted-out fractions of plasma.

4. The limitations of the process and the bearing of methods of immunization on yields and purity (u./g.) are discussed.

I am indebted to Dr J. W. Trevan and Dr C. G. Pope for their help and advice both during the period in which the work was carried out and in the preparation of the paper; also to Mr A. T. Glenny and his staff who carried out many of the tests for antitoxic potency. I am very grateful to Dr B. C. J. G. Knight for his criticism and help in the preparation of the manuscript for publication.

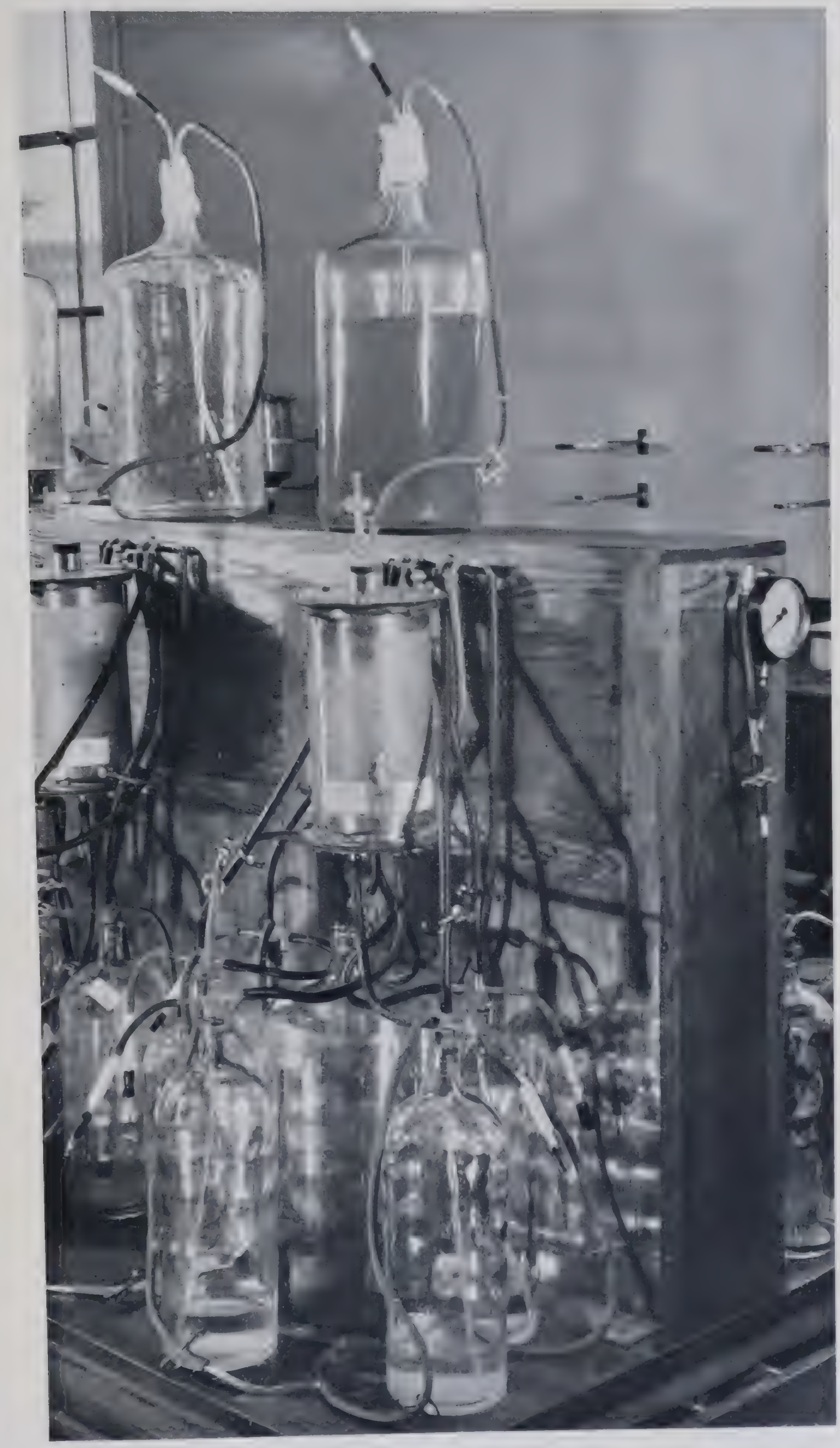

Fig. 3. Photograph showing the ultrafiltration in progress.

A. J. HARMS—THE PURIFICATION OF ANTITOXIC PLASMAS BY ENZYME TREATMENT AND HEAT DENATURATION

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Fructose and Glucose in the Blood of the Foetal Sheep

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Little is known of the distribution of D-fructose in animal tissues, apart from its demonstration in certain embryonic fluids (see, e.g., Needham, 1931, 1942). Recently it has been positively identified by Mann (1946) as the reducing sugar in the seminal plasma; the fact that hitherto it had been taken for granted that this was glucose suggests that fructose may have escaped detection in other instances.

There is no excuse for this, for a characteristic, though not entirely specific, colour reaction, due to Seliwanoff (1887), has been available, and in the methylphenylosazone Neuberg (1902; Neuberg & Strauss, 1902) found a derivative by which it could be distinguished from accompanying glucose, provided always that a sufficiently concentrated solution was available. Our own attention was drawn to the problem of identifying fructose by the late Sir Joseph Barcroft and Mr S. W. Cole, who in the course of recent work on the foetus of the sheep had confirmed an observation of Paton, Watson & Kerr (1907). The latter authors detected by the Seliwanoff test a fructose-like substance in the amniotic and allantoic fluids of sheep, cow, pig, cat, dog, rabbit, ferret and guinea pig, and in foetal blood. They investigated the blood more closely: 'by extracting large quantities of foetal blood [of the sheep?] with alcohol, evaporating with charcoal, and taking up in a small quantity of water, we were able to extract a sugar which was laevorotatory and which gave Seliwanoff's reaction distinctly...'. Orr (1924) obtained similar results using lead acetate instead of charcoal, with blood from a newly born kid, and blood taken from the human placenta after birth.

The observations of Cole & Hitchcock (1946) indicated that appreciable quantities of fructose (up to 100 mg./100 ml.) were present in the blood of the foetal sheep, and at Sir Joseph Barcroft's request we attempted (*a*) to prove that the substance responsible for the colour reaction was in fact fructose, and (*b*) to isolate a derivative of the sugar.

EXPERIMENTAL

Methods

Standard fructose and glucose solutions. These were made in saturated benzoic acid solution (Folin, 1929); a concentrated solution (*c.* 2%) was made for polarimetric measurement of the concentration, and this was diluted with more saturated benzoic acid solution to give a final sugar concentration of 0·5 mg./ml. In our experience a solution of either sugar at this, or a lower concentration, does not deteriorate when kept for several years at room temperature in a well-stoppered bottle.

Total reducing substances (t.r.s.). (More strictly, reducing substances estimated by the ferricyanide-cerium method.) The procedure used was that of Miller & Van Slyke (1936), the solutions being heated in boiling tubes without stoppers, and titrated to the nearest drop (0·02 ml.) after cooling and acidification, using xylene cyanole FF as an internal indicator. Instead of standardizing the ceric sulphate solution and using a fixed time of heating, a standard fructose, and occasionally a glucose solution, containing 100 μg. of sugar, was included in each set of estimations, and heating was carried out for 10–12 min. in a boiling water bath. The ceric sulphate solution was of such a concentration that 1 ml. was equivalent to about 100 μg. of reducing substance, calculated as hexose. Contrary to the findings of Miller & Van Slyke (1936) we found no discrepancy greater than the experimental error, between the reducing values of glucose and fructose, nor did we find any appreciable variability in the reducing value of fructose.

Fructose. For the estimation of those substances giving the Seliwanoff reaction, called here 'fructose' for the sake of brevity, a modification of Roe's method (1934) was used. This was devised by Mr S. W. Cole, who very kindly gave us the details and provided the facilities for carrying it out. A full account of the application of the method to the estimation of fructose and inulin in blood and urine will be published at a later date.

To the test material contained in 4 ml. of aqueous solution (in the case of the blood filtrates, diluted 1 in 10, 1 ml. plus 3 ml. of water was usually sufficient), in glass-stoppered test tubes, were added first 6 ml. of resorcinol solution (A.R., 0·15% (w/v) in absolute ethanol), thereafter at 1 min. intervals 6 ml. of conc. HCl containing 7·5 mg. $FeCl_3$/l. After thorough mixing of the contents, the tubes were placed at 1 min. intervals in a thermostatically controlled water bath at 75°, and left for 30 min. They were then cooled in jars of cold water, and the colour developed read with suitable filters in 2 cm. cells in a Spekker photoelectric absorptiometer, against a 'blank' on the reagents plus water. Standard fructose solutions containing 50 or 100 μg. were included in each set of estimations; these gave densities of about 0·32 and 0·65 respectively. The majority of readings were made with a spectrum blue filter (Ilford Ltd., no. 602), but in some experiments a Wratten no. 75 (Kodak Ltd.), which gave rather smaller densities, was used instead. (In a very few experiments the bath temperature was 80° and the time of heating then either 20 or 30 min.) Mr Cole tells us that under these conditions the specificity of the method is such that glucose gives between 0·6 and 0·8% of the colour given by an equal amount of fructose, and that the relationship of density to concentration over the range 20–100 μg. is for all practical purposes a straight line.

Yeast fermentation. This was carried out with a commercial (Distillers Company Ltd.) baker's yeast. A sample of the pressed yeast was suspended in ten times its weight of tap water, left for an hour or more at room temperature, centrifuged, and resuspended in water. In some experiments the washing was repeated; in others the yeast was resuspended in phosphate buffer at pH 6. An equal volume of the suspension was added to the test solution and left, with occasional mixing, for three or more hours at room temperature, or for a shorter period at 25°. Under these conditions, as the results below show, fructose is completely removed from the solution.

Estimation of glucose by the use of glucose oxidase. For these estimations we are greatly indebted to Dr T. Mann (cf. Mann, 1946) who provided a solution of the enzyme and assisted in the carrying out of the experiments. The oxidation was allowed to proceed at room temperature in the presence of a trace of catalase, after which appropriate estimations were made, usually without preliminary deproteinization.

Source and collection of blood

All the foetal blood used was obtained for us by Sir Joseph Barcroft, and was collected from a cannula in the carotid artery of the foetus, or, occasionally, by a syringe from the umbilical vessels, when the maximum quantity was wanted. The foetus was in every case still attached by the placenta to its mother who was anaesthetized with spinal Duracaine before the operation, and both were partly immersed in a warm bath. Observations had been made on the foetus and mother during the 1–1·5 hr. between the opening of the uterus and the taking of blood, but these were not considered to have disturbed the metabolism of the foetus unduly. It is likely, however, that the blood sugar level of the mother was in these circumstances higher than before anaesthetization (Hitchcock, 1948), and the glucose level in some of the foetal blood samples may have been raised correspondingly.

The blood was allowed to run through a rubber tube into a convenient volume of distilled water (see below) contained in a stoppered measuring cylinder which had been chilled on ice, the time for collection, even of large samples, not exceeding 5 min. In this way glycolysis was reduced to a minimum. The partly haemolyzed blood was mixed thoroughly, the increase in volume noted, and samples withdrawn for cadmium deproteinization (see below). The amount of blood collected was inferred from the increase in volume. Immediately after the withdrawal of samples more water and 97% ethanol were added, and the haemolysis thereby completed. The mixture was then transferred to bottles of litre capacity stoppered by rubber bungs, and kept cold until the next step in solvent deproteinization.

Deproteinization

With cadmium hydroxide. This was used for the samples of diluted blood, which were used to measure the t.r.s. and fructose contents of the blood, and as a check on the recovery of the sugars in the bulk deproteinization by organic solvents. The method used was essentially that of Fujita & Iwatake (1931). The precipitated material was removed by centrifuging because of the possibility that heating in alkaline solution might convert glucose to fructose. The supernatant fluids obtained were water-clear; for convenience they are referred to as 'cadmium filtrates'.

With ethanol-chloroform. This method is an adaptation by Keilin & Mann (1940) of that used by Tsuchihashi (1923), and we are grateful to Dr Mann for the guidance which he gave us in its use. A typical treatment was as follows: 160 ml. of blood were collected into 200 ml. of water. After withdrawal of two 1 ml. samples for cadmium deproteinization, haemolysis was completed by the addition of a further 250 ml. of water, and 300 ml. of 97% ethanol. To the haemolyzed mixture 150 ml. of chloroform were added and the mixture shaken *very gently.* The resulting precipitate did not flocculate completely. 2 ml. of approx. 20% acetic acid were added to make the suspension orange to methyl red, and after very gentle agitation the precipitate separated rapidly, leaving a water-clear supernatant fluid. The material was left in the refrigerator for 2 hr. and centrifuged. (Centrifuging at this stage gives three layers, an upper aqueous one, an intermediate layer of coagulated protein, and a lower layer of chloroform. When the aqueous layer is decanted the chloroform sometimes runs out as well, but can easily be separated from it.) 755 ml. of supernatant fluid were decanted through glass wool. The protein and chloroform layers were shaken up vigorously with 300 ml. of an emulsion made by shaking a 2 : 1 water-ethanol mixture with an excess of chloroform. The suspension was again centrifuged and 300 ml. of water-clear supernatant fluid decanted through glass wool into the main bulk. The colourless solution was stored in the refrigerator.

In our experience it was necessary to adjust the pH carefully, and not to shake too vigorously at any stage, otherwise the coagulated protein formed a fine suspension which made opalescence in the aqueous phase difficult to see. Ideally, a water-clear fluid should be obtained on centrifuging; slight opalescence, however, did not appear to interfere with subsequent operations. For convenience, the deproteinized solutions obtained by this procedure will be referred to as 'ethanol-chloroform filtrates'.

Concentration of ethanol-chloroform filtrates. This was done under reduced pressure with a bath temperature not exceeding 50°. When the volume had been reduced to about 20 ml., representing usually a fivefold concentration of the original blood, solid separated, but filtration in the presence of kieselguhr or Hyflo Supercel readily gave a clear pale-yellow solution. Alternatively the last stages of evaporation were done in the presence of a relatively large volume of Hyflo Supercel (e.g. 2 g.) and the blood concentrate was thereby obtained in a dry finely divided form. In a few experiments the concentrate was taken to complete dryness and extracted with absolute ethanol (see below). (All these are called 'ethanol-chloroform concentrates'.)

Recoveries

Fructose. A preliminary experiment showed that fructose added to maternal blood could be recovered in good yield in the ethanol-chloroform filtrate. The comparison of fructose contents of ethanol-chloroform filtrates and cadmium filtrates prepared from the same samples of foetal blood showed that the recovery at this stage was nearly complete (Table 1).

T.r.s. Recovery of t.r.s., though less significant, indicates no great discrepancy between the two methods of deproteinization (Table 1).

The reducing substances in cadmium filtrates of triplicate diluted samples of blood 19 were analyzed for us by Mr M. W. S. Hitchcock, who used the copper-reduction method of Somogyi (1945). Corrected for dilution, these gave an average of 133·3 mg. reducing substances/100 ml. of blood, as against our value of 130·4 mg./100 ml. by the ferricyanide-cerium method.

Ethanol extraction. Table 2 shows the percentage recoveries (in all fractions) of fructose and t.r.s. at several stages of the working up of a sample of the pooled blood filtrates 3, 4, 5, 6. The losses on removal of the ethanol and chloroform are probably mainly fictitious, since these solvents increase slightly the values obtained by both methods (e.g. in the fructose estimation the addition of 1 ml. of the clear supernatant fluid from shaking together 10 ml. water, 5 ml. 97% ethanol, and 2 ml. chloroform increased the 'fructose' value of 50 μg. fructose to 52·4 μg., and of 100 μg. to 103·2).

From these results it was concluded that the ethanol-chloroform filtrates could be taken as reasonably representative of the original bloods, in so far as fructose and t.r.s. were concerned.

RESULTS

Composition of the original foetal blood samples as measured on the cadmium filtrates

The figures in Table 3 give the age of the foetus, and the fructose and t.r.s. contents of the blood sample, measured on the cadmium filtrate (cf. Cole & Hitchcock, 1946). In some cases the amount of blood used was not the whole of that obtained from the foetus. It will be seen that variation in the fructose and t.r.s. contents is apparently at random, although Hitchcock (1948), who took samples from younger foetuses, suggests that the fructose content falls towards the end of term (147 ± 3 days), while the t.r.s.-fructose (which he assumes to be glucose) rises.

Table 1. *Total reducing substances (t.r.s.) and fructose (F) in ethanol-chloroform and cadmium filtrates compared*

	Amount in sample (mg.)							
Blood sample ...	3, 4, 5, 6*		3, 4, 5, 6, 7*		11		19	
	T.r.s.	*F*	T.r.s.	*F*	T.r.s.	*F*	T.r.s.	*F*
Cadmium filtrate	147	30	105	56	326	158	196	124
Ethanol-chloroform filtrate (evaporated to small bulk)	157	28	98	52	311	137	201	104

* Pooled ethanol-chloroform filtrates from several blood samples; the figures refer to a portion of the combined filtrates. *F* = fructose.

Table 2. *Recovery of fructose and t.r.s. at successive stages of concentration and ethanol extraction of an ethanol-chloroform filtrate*

	Recovery of fructose (%)		Recovery of t.r.s. (%)	
Stage	Between stages	Overall	Between stages	Overall
Initial ethanol-chloroform filtrate	—	(100)	—	(100)
Evaporated to dryness *in vacuo* below 50°	94·8	94·8	88·3	88·3
Extracted with boiling absolute ethanol	86·2	81·8	97·3*	86·0
Ethanolic extract evaporated to dryness *in vacuo*	97·7	80·0	89·9	77·3

* Part in extract, part in residue.

Table 3. *Composition and other data on blood samples, measured on cadmium filtrates*

No.	Age of foetus (days)	Fructose (mg./100 ml.)	T.r.s. (mg./100 ml.)	Vol. used (ml.)	No.	Age of foetus (days)	Fructose (mg./100 ml.)	T.r.s. (mg./100 ml.)	Vol. used (ml.)
1	107	142	157	70	11	142	71	145	224
2	112	97	—	60	12		75	155	300
3	120	123	142	62	13	141	51	83	184
4*	108	53	84	69	14†	142	56	114	142
5	129	34	61	71	15		76	150	180
6		25	57	85	16	141	69	107	160
7	132	69	159	178	17†	141	69	109	250
8†	139	66	—	110	18	124	—	—	50
9†		81	—	178	19	141	82	130	150
10	144	35	86	—	20	—	55	82	—

* Twin died, not sampled. — Not estimated.

† 10–70 ml. of blood taken for other purposes. The bracketed foetuses are twins.

Analysis of concentrates of ethanol-chloroform filtrates

(*a*) *Optical rotation.* This was first measured on an aqueous solution of the absolute ethanol-soluble fraction of the material in an ethanol-chloroform filtrate. In a 2 dm. tube the rotation was −0·73°; that calculated from its fructose content was −0·52°, suggesting that other laevorotatory substances were present, and that they were ethanol-soluble. Subsequently, rotations were read of several ethanol-chloroform concentrates; these are summarized in Table 4 with the values calculated from the fructose contents.

Table 4. *Rotations of concentrates from ethanol-chloroform filtrates compared with the rotations calculated from their fructose contents*

Blood sample ...	3, 4, 5, 6, 7	11	19	12, 14, 16, 37
	(α_D)	(α_D)	(α_D)	(α_D)
Rotation in 2 dm. tube	−0·58°	−1·54°	−1·40°	−2·10°
Rotation calculated from fructose content	−0·32°	−1·00°	−0·75°	−1·42°

These results indicate clearly the presence of laevorotatory material which is not fructose, and illustrate the danger of assuming that laevorotation in itself is evidence for the presence of fructose (cf. Orr, 1924).

(*b*) *Change in optical rotation on treatment with yeast.* If the other laevorotatory material were not yeast-fermentable it should be possible to show that the change in rotation accompanying fermentation is equivalent to the fructose disappearing. To this end a series of experiments was performed. It was essential to begin with a relatively large optical rotation, and fortunately the concentrates of ethanol-chloroform filtrates were almost colourless, and could be read after filtration without the need for decolorization.

In most experiments 6·0 ml. of the concentrate were mixed with 5·0 ml. of a yeast suspension prepared as described above, and the fermentation was allowed to go to completion. The yeast was then centrifuged out, leaving sufficient clear supernatant fluid for the rotation to be read in a 2 dm. tube (capacity *c.* 9·5 ml.). Estimations of fructose were done on the solution before and after fermentation, and the rotation due to the fructose fermented was calculated. When these experiments and those with glucose oxidase indicated the possible presence of glucose, fermentable t.r.s. were estimated also, and the difference between this result and the fructose content taken as glucose; the change in rotation expected on its disappearance was calculated and subtracted from that for the fructose. The results are summarized in Table 5.

These results make it clear that non-fermentable laevorotatory material is present. They also show fairly good agreement between the observed change in rotation and that calculated on the assumption that the fermentable reducing substance is a mixture of fructose and glucose. It will be noticed that in the absolute ethanol-soluble fraction the amount of non-fructose fermentable material is smaller, probably because of the limited solubility of glucose in that solvent.

Table 5. *Effect of yeast treatment on the optical rotation of ethanol-chloroform concentrates: comparison of the observed change in rotation with that calculated from the fructose and t.r.s. disappearing*

		Losses on fermentation				Optical rotation		Change	
		Fructose		Non-fructose					
Sample	Vol. (ml.)	(mg.)	(α_D)	(mg.)	(α_D)	Before	After	Observed	Calculated
11*	25	134·6	−1·00°	115·6	+0·49°	−1·54°	−0·97°	+0·57°	+0·51°
19	25	100·2	−0·75°	13·0	+0·06°	−1·40°	−0·67°	+0·73°	+0·69°
Control	10	120·1	−1·48°	58·6	+0·41°	−1·11°	0·0°	+1·11°	+1·07°
Ethanol extract 11†	25	112·0	−0·82°	15·4	+0·07°	−0·98°	−0·31°	+0·67°	+0·75°

* 10 ml. of the concentrate used for this experiment were evaporated to dryness *in vacuo* below 50°, pH 5, in the presence of 0·5 g. barium acetate and a little acetic acid. The residue, dried exhaustively over H_2SO_4, was extracted with a total of about 100 ml. absolute ethanol, the extract being filtered through Hyflo Supercel on a G4 filter and evaporated to dryness, with the same precautions as to temperature. The residue was extracted with exactly 14 ml. of approx. 2% Na_2SO_4, the $BaSO_4$ being removed by centrifuging. The clear supernatant liquid was used for the second experiment (†).

(*c*) *The action of glucose oxidase on concentrates.* Positive evidence for the presence of glucose was obtained through experiments with glucose oxidase.

A mixture of five ethanol-chloroform filtrates (500 ml.) (3, 4, 5, 6, 7) was evaporated to small bulk under diminished pressure with a bath temperature never higher than 52°. The pH was checked by means of methyl red throughout the concentration, which took 5 hr. A little kieselguhr was added and the suspension filtered by pressure on a G3 sintered-glass filter. The volume of filtrate was made to 30 ml. Samples (2·0 ml.) of this were taken for the estimation of yeast-fermentable t.r.s. and fructose. Other 2·0 ml. samples were taken to study the effect of glucose oxidase (plus catalase) on the t.r.s. and fructose. 200 μg. of enzyme were used in a total volume of 2·9 ml., which included 0·5 ml. of M-acetate buffer (pH 5·2). The solutions were shaken for 1·5 hr. in the cups of Barcroft manometers. Estimations of t.r.s. and fructose were done without deproteinization. A control on the enzyme and buffer was not distinguishable from the blank, and the buffer had no effect on the results of either estimation when added to 100 μg. of fructose. The significant findings are summarized in Table 6.

Table 6. *Effect of glucose oxidase and yeast on fructose and t.r.s. contents of a pooled ethanol-chloroform concentrate*

(All results expressed as mg./30 ml. of the original concentrate.)

	Untreated concentrate		After glucose oxidase treatment		After yeast treatment
	Alone	With buffer	No addition	2 mg. glucose added	
Fructose	50·9	51·6	51·0	50·3	1·2
T.r.s.	97·3	98·6	73·7	73·0	22·3

These results demonstrate that the enzyme had no effect on fructose, and that it had worked to completion. From them were calculated:

	(mg.)
(1) Fermentable reducing substances	(97·3–22·3) = 75·0
(2) Fermentable fructose	(50·9–1·2) = 49·7
(3) Fermentable non-fructose reducing substances	(75·0–49·7) = 25·3
(4) Glucose from the effect of glucose oxidase	(98·6–73·7) = 24·9

The agreement between (3) and (4) suggests that all the fermentable reducing substances can be accounted for by fructose and glucose.

A repetition of this experiment with blood sample 19, in which all the solutions were deproteinized before estimations were done, gave rather less satisfactory agreement; the ratio of fructose to glucose in this sample was much higher, about 8 : 1.

(*d*) *Other constituents of the concentrates.* The concentrate used for the experiment of Table 6 contained 82 mg. of lactic acid in 30 ml., corresponding to 91 ml. of mixed blood. (This is not enough to account, as L-lactate, for the non-fermentable laevorotatory material; see (*b*) above, cf. Bell, 1932.) In addition the concentrates gave a strong nitroprusside reaction which was removed by cadmium deproteinization. However, there is evidence that this treatment does not remove all the non-fermentable reducing material (see below). It was thought that extraction with absolute ethanol in the presence of barium acetate might leave behind some of these substances, yet the extract so obtained from blood sample 11 still contained 50 mg. of non-fermentable t.r.s. ('fructose' content, 4·2 mg.) as against 56·5 before this treatment. The fermented extract still had a strong negative rotation (see Table 5).

We are indebted to others for certain additional information: Miss V. Moyle kindly estimated allantoin for us, in blood concentrate 19, by the method of Young, MacPherson, Wentworth & Hawkins (1944), and found an

amount corresponding to 0·3 mg./100 ml. of the original blood (cf. 1·9 mg./100 ml. in adult sheep's blood, and similar values for cattle and pigs; Fosse, Brunel & Thomas, 1931). Dr S. M. Partridge (Partridge, 1948) has shown that sample 18, and pooled samples (3, 4, 5, 6, 7, 13, 15) and (8, 9, 10) contain inositol. Inositol gives no reduction under the conditions of the ferricyanide-cerium method for t.r.s.

Isolation of fructose derivatives

Methylphenylosazone. A preliminary experiment on maternal blood with added fructose showed that the osazone could be prepared from ethanol-soluble material in an ethanol-chloroform filtrate. This procedure was applied to the first sample of foetal blood.

The ethanol-soluble fraction (0·6 g.) of the material in the ethanol-chloroform filtrate, estimated to contain 57 mg. of fructose, was dissolved in 2·5 ml. of water, in which it was completely soluble, 1 ml. of freshly distilled methylphenylhydrazine added, with 2·2 ml. of 96% ethanol to render the mixture homogeneous, and finally 1 ml. of 50% acetic acid. The darkening solution was warmed in a water bath for 10 min. and left at room temperature for 1·5 hr. 10 ml. of N-acetic acid were then added, and the osazone extracted with successive portions of 20, 15 and 15 ml. of chloroform. The combined extracts were shaken with 25 ml. of N-acetic acid, then with dilute $NaHCO_3$ solution until no more CO_2 was given off, and finally with water. After being dried over anhydrous Na_2SO_4, the chloroform solution was boiled with a little charcoal, filtered, and evaporated to dryness under reduced pressure. The 0·8 g. of residue (*N*-methylaniline was present) was recrystallized from a mixture of chloroform-ligroin (b.p. 40–60°). The melting point was raised to 151–152° by recrystallization from 20% ethanol-water. Yield 37·8 mg. (31% of theory). Under the same conditions 134·5 mg. of pure D-fructose gave 84·6 mg. of osazone (29% of theory). 170 mg. of glucose gave no crystallizable product.

The tetraacetyl derivative was prepared from the osazone from foetal blood: 23·2 mg. were dissolved in 2 ml. of 2 : 1 pyridine-acetic anhydride mixture and left 20 hr. at 40°. 20 ml. of water were added, the mixture shaken vigorously, left 4–5 hr. and filtered. The thoroughly washed material was dissolved in the minimum of hot ethanol, and water was added at boiling point until crystallization began. Yield 27·9 mg. of lemon yellow crystals; m.p. 125–126° (cf. Percival & Percival, 1937).

The yields of the methylphenylosazone are much lower than those claimed by Neuberg in his earlier papers. The highest which we have obtained by following his methods exactly are about 50% of theoretical. We have not applied the new modification of the procedure which he published after the present work was completed (Neuberg & Mandl, 1946).

The diacetone compound. Following our experience of the somewhat unsatisfactory osazone derivative, one of us (D.J.B.) has devised a method by which fructose can be isolated in high yield as its 2:3-4:5-di-*iso*propylidene derivative (Bell, 1947).

The procedure adopted for use with the deproteinized blood was as follows (all concentrations and evaporations were done under reduced pressure, below 35°). The ethanol-chloroform filtrate (1 to 5 l.) was evaporated to small bulk, 2 g. of Hyflo Supercel added and evaporation continued, the solid residue being dehydrated by distillation with ethanol-benzene, and finally kept for some hours in a high vacuum over H_2SO_4 and KOH. The dry mass resulting from the above procedure was broken up, if necessary, and shaken with 50 ml. of acetone (dried over $CaCl_2$) to which had been added 2·5 ml. of conc. H_2SO_4. After 6 hr. anhyd. Na_2CO_3 was added with vigorous stirring until the original dark brown colour of the fluid had changed to a very pale yellow. The solids were filtered off, washed with acetone, and the combined filtrates evaporated to small bulk in the presence of a few drops of 40% NaOH solution. (This ensures that acidic condensation by products of acetone and H_2SO_4 do not attack the labile *iso*propylidene groups in the sugar derivatives.) 100 ml. of water were then added to the syrupy residue and the whole distilled until all the mesityl oxide (a by-product) had passed over. The residue in the flask (*c.* 50 ml.) was shaken with a little charcoal to remove insoluble acetone by-products and filtered into a separating funnel. The filtrate was three times extracted with its own volume of chloroform, the extract dried over anhyd. Na_2SO_4 and evaporated to dryness. The residue consisted of 2:3-4:5-diacetone fructose, a little of its 1:2-4:5-diacetone isomer, and 1:2-5:6-diacetone glucose. The former compound is completely stable to 0·1 N-H_2SO_4 at room temperature, while the two latter substances undergo quantitative partial hydrolysis to the 1:2-monoacetone derivatives. The mixture was therefore dissolved in cold 0·1 N-H_2SO_4 and the solution kept for 6 hr., after which time it was rendered alkaline (pH 9) by addition of 40% NaOH solution, shaken with charcoal if deemed necessary, and filtered into a separating funnel. Threefold extraction of the filtrate with its own volume of chloroform quantitatively extracted the 2:3-4:5-diacetone fructose only. After drying over Na_2SO_4 the extract was evaporated to dryness and the resulting crystalline residue recrystallized from ligroin (b.p. 60–80°).

The results of three experiments are summarized in Table 7.

Table 7. *Yields of diacetone fructose from ethanol-chloroform concentrates*

Blood samples	Vol. of filtrate (ml.)	Fructose content of blood sample (mg.)	Crude yield of diacetone compound		After recrystallization	
			(mg.)	% of theory	m.p.	$[\alpha]_D$ (H_2O)
8, 9, 10	1200	136	140	71	94–95°	−32·95°
3, 4, 5, 6, 7, 13, 15	2500	280*	353	87	95°	−32·80°
12, 14, 16, 17	4900	470†	480	71	95°	−32·90°

Fructose : non-fructose ratios in cadmium filtrates: *1·3 : 1, †1·2 : 1.

Bearing of these results on the analyses of cadmium filtrates

Relation of results on ethanol-chloroform concentrates to those on cadmium filtrates. The main difference between the two types of deproteinization is in the degree to which non-fermentable reducing substances are removed. The non-fermentable reducing substances in the ethanol-chloroform filtrates are a considerable fraction of the t.r.s. With human blood and adult sheep blood the cadmium method removes effectively all this material, which includes such substances as glutathione, uric acid, etc. The question therefore arises as to the validity of assuming that the same is true of the foetal blood, and that the difference between fructose by Seliwanoff, and t.r.s. by ferricyanide-cerium is to be attributed to glucose.

That this might not be the case was shown by the fructose : glucose ratios obtained by Seliwanoff and glucose oxidase with ethanol-chloroform concentrates of bloods 3, 4, 5, 6, 7 (pooled) and 19. In the first this was rather greater than 2 : 1 (52·0 : 24·3) and in the second 8 : 1 (104·1 : 13·2). The fructose : non-fructose t.r.s. ratios of the cadmium filtrates were respectively 56·0 : 48·8, and 123·6 : 39·2. In each case the recoveries of fructose are good (see Table 1), and the discrepancy could be due either to an incomplete recovery of glucose in the ethanol-chloroform filtrate, or to the presence of non-fermentable reducing substances in the cadmium filtrate. The latter possibility was tested directly on other blood filtrates.

Glucose oxidase treatment and yeast fermentation of cadmium filtrates. It was found in preliminary experiments that both glucose oxidase and yeast would work in the presence of cadmium filtrates to which phosphate buffer had been added.

For these experiments blood was collected into a glass-stoppered flask containing potassium oxalate. 10, 5 and 1 ml. samples were withdrawn, treated with 80, 40 and 8 ml. of acid cadmium sulphate solution, and shortly afterwards with 10, 5 and 1 ml. of NaOH, of a concentration adjusted to give complete precipitation of the cadmium. The 1 ml. sample was heated in a boiling water bath for 2 min., cooled and filtered. The other two were centrifuged and the clear supernatant liquid decanted through filter papers into stoppered flasks. 5 ml. of 0·5 M-phosphate buffer (pH 6) were diluted to 50 ml. with the unheated cadmium filtrate, and used for the experiments in Table 8. No precipitation was noticed in this solution, which remained perfectly clear on standing for several hours. A second series of experiments was done to compare the heated and unheated cadmium filtrates, particularly with respect to non-fermentable reducing substances. The results of these are summarized in Table 9.

The discrepancy between the t.r.s. estimations on the filtrate with and without added glucose (6, 4), indicates that the glucose oxidase had not worked to completion in the former, and the figures for glucose in the filtrate must therefore be regarded as a minimum. Unfortunately no more foetal blood was available for a repetition of the experiment, this being the last foetus of the 1946–7 season. This was not thought to justify delaying publication for another year, although it detracts from the value of the results here recorded.

Table 8. *Effect of glucose oxidase and yeast on fructose and t.r.s. contents of a cadmium filtrate of blood sample* 20

No. of exp.	Blood filtrate + buffer (ml.)	Water filtrate + buffer (ml.)	Yeast suspension (ml.)	Glucose oxidase + catalase (ml.)	Added sugar	Fructose (mg./100 ml. blood)	T.r.s. (mg./100 ml. blood)
1	—	—	—	—	—	55·2	82·4
2	20·0	—	10·0	—	—	1·1	14·8
3	—	20·0	10·0	—	2 mg. fructose	1·1	8·0
4	20·0	—	—	0·2	—	54·5	63·6
5	—	20·0	—	0·2	—	—	3·4
6	5·0	—	—	0·05	1 mg. glucose*	57·2	76·0

* The added glucose would represent an additional 200 mg./100 ml. of the original blood, i.e. about 10 times that in 4.

Table 9. *Effect of heating cadmium-precipitation mixture on the amount of non-fermentable t.r.s.*

No. of exp.	Cadmium filtrate of blood (ml.)	Cadmium filtrate of water (ml.)	Yeast suspension (ml.)	0·1 M-Phosphate buffer, pH 6 (ml.)	Fructose (mg./100 ml. blood)	T.r.s. (mg./100 ml. blood)
7	5·5*	—	3·0	1·0	2·5	26·4
8	—	6·0	3·0	1·0	—	14·0
9	6·0	—	3·0	1·0	1·4	23·8

* Heated 2 min. at 100°; plus 0·5 ml. water.

From these results the following calculations can be made:

The relation between non-fructose fermentable t.r.s., and glucose by glucose oxidase.

From (1), (2), (3) we have 82·4 − (14·8 − 8·0) = 75·6 mg./100 ml. fermentable t.r.s.

From (1), (4), (5) we have 82·4 − (63·6 − 3·4) = 22·2 mg./100 ml. glucose.

From (1), (2), (3) we have 55·2 − (1·1 − 1·1) = 55·2 mg./100 ml. fermentable fructose.

I.e. non-fructose fermentable t.r.s. 75·6 − 55·2 = 20·4 mg./100 ml. Glucose by glucose oxidase = 22·2 mg./100 ml.

The difference between these two figures (1·8 mg./100 ml. of the original blood) is within the limits of experimental error.

The non-fermentable t.r.s. in the cadmium filtrate. This is equivalent (as hexose) to 6·8 mg./100 ml. (2, 3), 12·4 mg./100 ml. (7, 8), and 9·8 mg./100 ml. (9, 8). These values, which are similar to those obtained in an earlier experiment, could well be disregarded in the estimation of t.r.s. in the cadmium filtrate, but they are of the same order as the glucose content, and 'inflate' this if it is assumed to be equivalent to (t.r.s. − fructose). This result confirms the earlier supposition based on experiments with ethanol-chloroform concentrates, and it would seem that when a critical investigation of the glucose and fructose levels is being conducted, the use of glucose oxidase or some other method specific at least for aldoses, is desirable.

DISCUSSION

The experiments here described establish beyond doubt that all, or practically all, the substance in the blood of the foetal sheep giving a positive Seliwanoff reaction is D-fructose. They further establish an equally important fact, that this fructose is present mainly in the free state. This could not be inferred from the Seliwanoff reaction, nor from the formation of the diacetone derivative, nor even from its removal by yeast, but is clear from the formation of a methylphenylosazone, and from the changes in optical rotation accompanying yeast treatment. We believe that this is the first unequivocal demonstration of the presence of free fructose in blood.

In addition, the experiments provide strong evidence that apart from free fructose the blood also contains free glucose, but in much smaller concentrations in most of the samples examined. Within the limits of the methods employed there was no evidence for the presence of any other fermentable reducing sugar. This is confirmed by the findings of Partridge (1948) with the paper partition chromatogram; he detected glucose and fructose, but no appreciable quantities of any other reducing sugar.

It will be noticed that neither the glucose nor fructose, nor the two combined, are apparently maintained at a constant level, as one is accustomed to find the blood sugar of some animals. This may be due in part to the fact that the samples were taken from anaesthetized animals (cf. Hitchcock, 1948).

The proof that fructose is a constituent of foetal blood helps to explain the finding of Passmore & Schlossmann (1938) that the blood sugar level of the foetal sheep was higher than that of its mother (also in the case of the goat, and in the cow and pig; Aron, 1924). It seems probable from our results, and those of Cole & Hitchcock (1946) that the blood *glucose* (which should be if anything less than the non-fructose t.r.s.) of the foetus is lower than that of its mother, which would be more in keeping with results in other animals (cf. Needham, 1931), although of course this is no reason for assuming that fructose does not occur in the foetal blood of these species also. Orr (1924) states that Paton *et al.* (1907) demonstrated the presence of fructose in the blood of both foetal sheep and cow, but we have not been able to find any direct reference to either animal in the original paper. Orr himself showed that there was a fructose-like substance in human foetal blood, and in that of a kid just after birth, but from the feeble negative rotation of his blood concentrates he concluded that more glucose than fructose was present.

By the use of the methods we describe it should be possible to identify and estimate fructose in a variety of foetal bloods, and thus discover whether its occurrence is general. It might be expected that this is so from the general occurrence of Seliwanoff-positive substances, almost certainly fructose, in the amniotic and allantoic fluids of a wide range of species. At birth it would seem that the fructose is quickly lost, a part, or all being eliminated in the urine (cf. Langstein & Neuberg, 1907; Cole & Hitchcock, 1946).

It is too early to attempt an explanation for the presence of fructose in the foetus, or, for that matter, for the equally obscure and abrupt transference of the young animal at birth to a diet containing lactose, although the suggestion of Mann (1946), that fructose provides a source of energy for spermatozoa which is not shared by the somatic tissues, may have some bearing on this problem also.

SUMMARY

1. The Seliwanoff-positive substances in the blood of the foetal sheep have been accounted for by the presence of free D-fructose: they are fermentable, have a negative optical rotation closely corresponding to that of D-fructose, yield a methylphenylosazone under conditions in which glucose forms none, and give the expected high yield (70 %)

of 2:3-4:5-di-*iso*propylidene-D-fructopyranose on treatment with acid acetone under suitable conditions.

2. The reducing substances other than fructose are partly fermented by yeast, and this fraction has been accounted for by D-glucose, which was estimated by the disappearance of reducing substances on treatment with the specific enzyme, glucose oxidase.

3. A small part of the reducing substances in solutions deproteinized by the use of acid cadmium sulphate and sodium hydroxide, and a greater part of those in solutions deproteinized by chloroform and ethanol, are not fermented by baker's yeast; no evidence was obtained for the presence of any reducing sugar other than D-fructose and D-glucose.

4. The implications of these findings are discussed.

We wish to place on record our gratitude to the late Sir Joseph Barcroft, F.R.S., for the opportunity to investigate this problem, and for his encouragement, given so generously and in so many ways. We are indebted to Dr T. Mann and Mr S. W. Cole, for their advice and help in the course of the work; to Miss V. Moyle, Mr M. W. S. Hitchcock, and Dr S. M. Partridge for analyses which they performed for us; and to the members of the Agricultural Research Council Unit of Animal Physiology for their friendly co-operation.

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Physicochemical Observations on some Glycogens

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The molecular weight of unsubstituted glycogen has, up to the present, been studied in but a few instances. Oakley & Young (1936) have reported, for samples from rabbit liver and muscle, values of $1{\cdot}2$–$2{\cdot}3 \times 10^6$ for the former, and $0{\cdot}7$–$1{\cdot}8 \times 10^6$ for the latter material. These results were obtained by use of a delicate method of osmotic-pressure measurement on solutions in 0·1 N-calcium chloride. These authors claim that both alkaline extraction of the tissue (the so-called Pflüger method) and water extraction followed by precipitation by acetic acid (Bell & Young, 1934) yield products of the same general molecular size. Oakley & Young (1936) further noted that glycogen solutions when salt-free manifested markedly greater osmotic pressures than in presence of Ca^{++} and Cl^-. Bridgeman (1942) determined the sedimentation and diffusion constants of rabbit-liver glycogen and derived from these an average molecular weight of about 4×10^6. Glycogen from tubercle bacilli has been shown by Chargaff & Moore (1944), using similar methods, to have a molecular weight of 12–13×10^6. These are the only reported molecular weight determinations using the ultracentrifuge.

Our object in the present work was to compare certain physical properties of a number of glycogen preparations.

EXPERIMENTAL AND RESULTS

Preparation of material. Preparations were made from the appropriate tissue either by water extraction or by the Pflüger technique. In each instance final purification was effected by fourfold precipitation by acetic acid (Bell & Young, 1934). Relevant data are summarized in Table 1.

Table 1. *Glycogen specimens examined*

Serial number	Source	Method of isolation (P, by Pflüger technique; W, by water)	No. of residues in average unit chain	References to previous work on specimen used
I	Horse muscle	P	11–12 11–13	Bell (1937) Bell (1944)
II	Rabbit muscle	P	12	Bell (1948)
III	Human muscle	P	11	Halsall, Hirst & Jones (1947)
IV	Rabbit liver	P	12	Bell (1935)
V	Rabbit liver	W	12	Bell (1935)
VI	*Ascaris lumbricoides*	P	12–13 13–14	Baldwin & King (1942) Bell (1944)

Table 2. *Molecular weights of glycogen specimens*

Specimen	Solvent	$\frac{\eta/\eta_0 - 1}{C}$	$D_{20} \times 10^7$	S_{20} (corr.) $\times 10^{13}$	Mol. wt.* from S_{20} and D_{20}	Mol. wt. from osmotic pressure
I	Water	0·074	—	—	—	—
	M-NaCl	—	1·50	63	$2{\cdot}9 \times 10^6$	—
II	Water	0·074	—	—	—	—
	M-NaCl	0·055	1·55	58	$2{\cdot}6 \times 10^6$	—
III	Water	0·060	—	—	—	—
	M-NaCl	—	1·65	57	$2{\cdot}4 \times 10^6$	—
IV	Water	0·120–0·065	—	—	—	$0{\cdot}5 \times 10^6$
	M-NaCl	—	1·27	81	$4{\cdot}4 \times 10^6$	$2{\cdot}0 \times 10^6$
V	Water	0·075–0·063	—	—	—	—
	M-NaCl	—	1·21	75	$4{\cdot}3 \times 10^6$	—
VI	Water	0·135	6·25	—	—	$0{\cdot}1 \times 10^6$
	M-NaCl	—	1·80	18	$0{\cdot}70 \times 10^6$	$0{\cdot}5 \times 10^6$

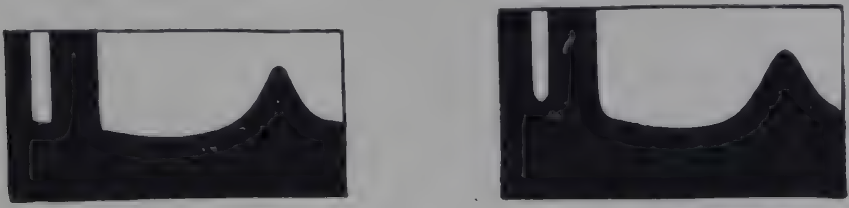
* Calculated from the equation mol. wt. $= \frac{RTS}{D(1 - V\rho)}$, where R = gas constant; T = absolute temperature; S = sedimentation constant; D = diffusion constant; V = partial specific volume and ρ = density of solvent.

Viscosity measurements. These were made in an Ostwald-type viscometer having a flow time for water of about 3 min. Series of solutions of each sample of glycogen, in concentrations ranging from 0·2 to 2·0%, were examined. Except where indicated in Table 2, we observed no marked dependence of the specific viscosity upon concentration.

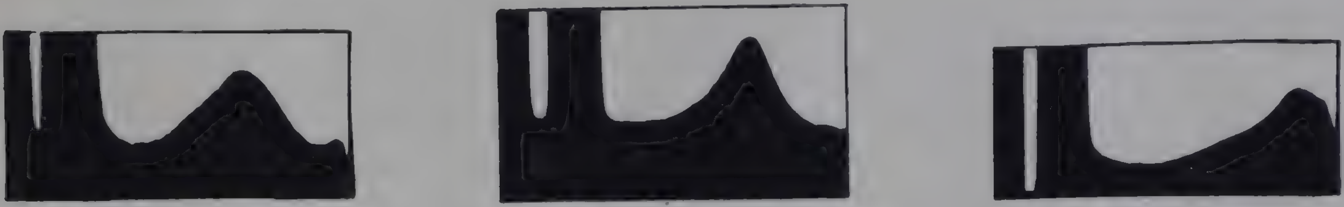
Diffusion rates. These were observed on 1% solutions (in M-NaCl) of the glycogens in a cell of the type described by Lamm & Polson (1936). Diffusion constants were calculated by the inflexion-point method and distribution curves over the boundary were obtained by the Philpot (1938) optical system. The diffusion constants (D_{20}) recorded in Table 2 are corrected to diffusion in water at 20°.

Sedimentation rates. The sedimentation velocities were observed in the Svedberg oil-turbine ultracentrifuge by the method of Philpot (1938). Fig. 1 shows specimen diagrams. The thicknesses of the sedimenting boundaries were in all instances much greater than could be accounted for by diffusion, showing that the samples were markedly polydisperse. The glycogen of rabbit liver prepared by the Pflüger method (sample IV) was more polydisperse than that made by water extraction (sample V). The mean sedimentation constants, corrected to the values for water at 20° (S_{20} corr.), are given in Table 2.

Partial specific volume. V was calculated both from the densities of aqueous solutions and from the density of dry glycogen samples using diethyl β-ketoglutarate. (This liquid was chosen for the reasons that it is not appreciably volatile, has relatively low viscosity and rapidly wets glycogen without dissolving it.) There appear to be no significant differences between the densities of glycogen samples from different sources, and the calculated partial specific volume was equal to 0·64–0·66.



I. Horse muscle II. Rabbit muscle III. Human muscle



IV. Rabbit liver (Pflüger) V. Rabbit liver (Bell & Young, 1934) VI. *Ascaris* glycogen

Fig. 1. Sedimentation diagrams. The following are the values of the speed of rotation (rev./sec.) and the time after reaching full speed at which the diagrams were obtained (roman numbers refer to the glycogen specimens: see Table 1). I, II, III: 450; 25 min. IV: 400; 30 min. V: 315; 60 min. VI: 625; 20 min. Concentration of glycogen: in each case 1 g./100 ml. of solvent.

Osmotic pressure. This was measured by the method of Adair (1925). The concentration of glycogen after equilibrium was determined polarimetrically. A series of experiments over a range of concentrations of from 1 to 7 % of solute showed that the apparent molecular weight calculated from osmotic pressure is markedly dependent on concentration. It was difficult to apply the extrapolation to zero concentration recommended by Adair & Robinson (1930) as the pressure exerted by dilute solutions was very small and the results obtained yielded, therefore, only rough approximations.

DISCUSSION

From the observations recorded in Table 2, it is clear that glycogens rank among the largest known metabolites, and that significant differences of molecular size occur according to the source from which the glycogen is obtained. Although all the samples were polydisperse and although the degree of polydispersity evidently depends on the method of isolation, their general uniformity suggests that these values may be valid for glycogen as it occurs in the cell.

The lower values of molecular weight obtained by osmotic measurement are to be expected in view of the polydispersity of the material, since osmotic pressure depends on the 'number average' while sedimentation and diffusion depend on the 'mass average' of molecular species.

The lowering of the osmotic pressure by salts, already noticed by Oakley & Young (1936), is difficult to account for. Disaggregation is unlikely in view of the lowering of viscosity produced by salt. A Donnan lowering is unlikely in view of the absence of ionizable groups in glycogen, though compensation of a charge-effect would account also for the lowering of viscosity. The effect of salt on viscosity might be accounted for by a decreased asymmetry of the molecule; this might be accompanied by a reduction of the number of its possible configurations which would account, on statistical grounds, for the reduction of osmotic pressure.

SUMMARY

1. The average molecular weights of samples of muscle glycogen have been shown, from sedimentation and diffusion data, to be of the order of $2{\cdot}4\text{–}2{\cdot}9 \times 10^6$.

2. Samples of liver glycogen have molecular weights of $4{\cdot}4\text{–}4{\cdot}3 \times 10^6$. Alkaline extraction of the glycogen from its parent tissue does not effect very serious degradation.

3. Glycogen from the whole tissues of *Ascaris lumbricoides* has a much smaller molecular weight, namely $0{\cdot}70 \times 10^6$.

4. Osmotic pressure measurements have been shown to lead to estimations of molecular weight considerably smaller than those afforded by sedimentation-diffusion methods.

5. In all instances the glycogens were found to be polydisperse with respect to molecular size.

6. The observations of Oakley & Young on the effect of salts on the apparent molecular weight by osmotic pressure have been confirmed.

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Partial Amino-acid Compositions of some Plant-leaf Protein Preparations: the Arginine, Histidine and Lysine Contents

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In an earlier publication (Lugg & Weller, 1944) the amide, tyrosine, tryptophan, cyst(e)ine (i.e. cystine plus cysteine calculated as cystine) and methionine contents of some extracted plant-leaf protein preparations were reported. These preparations, one each from leaves of *Phalaris tuberosa* L., *Hordeum murinum* L., *Medicago sativa* L., and *M. denticulata* Willd., were judged, both from the mode of preparation and from analytical evidence, to be reasonably representative of the whole proteins in the leaf materials. The arginine, histidine and lysine contents of these same preparations have now been estimated and it is one purpose of this article to record the values obtained. The question of the existence of hydroxylysine in these preparations has also been considered.

EXPERIMENTAL

Methods. The arginine and lysine contents of the preparations were estimated by methods based essentially upon those of Tristram (1939). Histidine contents were at first estimated by Tristram's (1939) method, but for reasons to be discussed, the resulting values were considered unsatisfactory and recourse was had to an adaptation of the method of Vickery & Winternitz (1944). A modification of the Kapeller-Adler (1934) colorimetric method of estimating histidine, recommended by Block (1937), was unsatisfactory in our hands.

Hydrolysis. In Tristram's experience the hydrolysis of plant-leaf protein preparations for the purpose of estimating the contents of these three amino-acids could be performed satisfactorily by heating the protein with about ten times its weight of 4M-H_2SO_4 for 18–22 hr. at the boiling point of the mixture (approx. 110°). Whilst it is unlikely that these conditions would secure the maximum 'net production of amide NH_3 plus amino-acid "amino" groups' (see Lugg, 1946), they may achieve almost complete liberation of these particular amino-acids from such proteins. In our work, about 2·7 g. of the air-dried preparation (about 2·5 g. dry wt.) were heated for *c.* 1 hr. with 25 ml. of 4M-H_2SO_4 at 100° and then at the boiling point of the mixture for 21–22 hr., which is the upper region of Tristram's (1939) range.

Estimation of arginine, histidine and lysine. The excessive variability of replicate histidine values estimated by Tristram's (1939) method and the extreme uncertainty of values obtained by the Conrad & Berg (1937) modification of Kapeller-Adler's (1934) method (tints of standard and test colour solutions frequently differed markedly) made it desirable to find a more satisfactory procedure. The Vickery & Winternitz (1944) method, in which the histidine is estimated gravimetrically as the di-3:4-dichlorobenzene sulphonate (histidine-DBS), was adapted for use in a general procedure for the estimation of arginine and histidine.

In this procedure insufficient Ag_2O and H_2SO_4 for precipitation of Ag_2SO_4 to occur were used, and larger quantities of $AgNO_3$ than are needed in Tristram's (1939) procedure, had to be employed. This and subsequent steps were taken to prevent the introduction of variable amounts of H_2SO_4 and/or HCl, in the presence of which we found the solubility of histidine-DBS to be appreciably increased. Urea was added in small quantities to decompose any nitrous acid which may have been present (from decomposition of nitrate by reducing substances in acid solutions). Tristram's procedure was then followed for the separation of the arginine- and histidine-silver compounds and for the isolation of the arginine and lysine as monoflavianate and monopicrate, respectively.

The histidine was liberated from the silver compound by treatment with H_2S (Vickery & Winternitz, 1944, used HCl) and was then precipitated as histidine-DBS in the manner described by these authors, but from a volume of 10 ml. and with 1·5 g. of the acid. The salt was recrystallized repeatedly from aqueous solutions of the acid until the recrystallization loss was the same as that found for the pure salt. To the final weight were added the recrystallization losses of the pure salt for every recrystallization. Purity was confirmed by melting-point determination. A correction for overall losses of +2·1 mg. of histidine N was made (see later references).

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The arginine monoflavianate was recrystallized repeatedly from water and the final weight of pure salt was corrected for the recrystallization losses. A correction for overall losses of +0·4 mg. N was made (see later references) in addition to the correction for solubility of the arginine-silver compound. This correction, which is much smaller than Tristram's, was applied also to results obtained by his unmodified method. Incidentally, Chibnall, Rees & Williams (1943) believed the earlier correction to be too high. Weights of crude salt obtained by Tristram's unmodified method were corrected for impurity by multiplying by a factor, 0·965, derived by recrystallizing samples obtained in several analyses and pooled samples from the other analyses.

The lysine monopicrate was tested for purity by fractional recrystallization of mixed samples and chromatographing the fractions; a general correction was then applied by multiplying the weights of crude material by 0·975. Tristram's (1939) correction for overall losses (+1·6 mg. N) was made.

Arginine, histidine and lysine were thus estimated by Tristram's (1939) methods (method 1), but histidine (and, in some instances, arginine) were estimated also by the procedures outlined above (method 2).

Losses of arginine, histidine and lysine in the analytical procedures. In the examination of losses, mixtures of the following quantities of amino-acids were used: approx. 0·12 g. L-arginine, 0·04 g. L-histidine, 0·12 g. L-lysine, 1·0 g. glycine, 0·5 g. DL-alanine and 0·5 g. DL-aspartic acid. These mixtures, dissolved in 25 ml. of 4M-H_2SO_4 solution, were (*a*) submitted to direct analysis, (*b*) 'hydrolyzed' at the boiling point for 21–22 hr. and then analyzed, (*c*) treated with 0·5 g. of L-arabinose before 'hydrolysis' and analysis.

Table 1. *Percentage losses of arginine* (0·12 *g.*) *and histidine* (0·04 *g.*), *when submitted with lysine* (0·12 *g.*), *glycine* (1·0 *g.*), *alanine* (0·5 *g.*) *and aspartic acid* (0·5 *g.*) *to the hydrolysis procedure in the absence and in the presence of arabinose*

Arabinose present (g.)	Losses (%)	
	Arginine	Histidine
0	0	0
0·50	16	4

From replicate (*a*) experiments were found the mean corrections for overall losses (previously given) for use with method 2. From replicate (*b*) and (*c*) experiments were derived the data shown in Table 1. In analytical procedures (such as these) which involve the separation of amino-acid salts of moderate solubility, losses might be expected to arise from any racemization suffered by the amino-acids. The results shown in Table 1, and those of Tristram (1939) for lysine, indicate that to whatever extent racemization might have occurred, recoveries of the amino-acids could not have been affected by it. Losses in absence of carbohydrate, estimated by other methods also, appear to be zero for all three amino-acids concerned (see Chibnall, 1946).

Tests with regard to the possible presence of hydroxylysine in the protein preparations

Van Slyke, Hiller, Dillon & MacFadyen (1938) have described the isolation of lysine and hydroxylysine picrates from gelatin hydrolysates by a procedure which differs very little from that used in this work (above) for the isolation of lysine monopicrate, the main points of difference being that they repeatedly recrystallized the phosphotungstates to remove other amino-acids, and fractionally precipitated the picrates. They state that hydroxylysine picrate is rather more soluble than the lysine salt in water. From their work it may be presumed that, if the leaf-protein preparations were to contain appreciable quantities of hydroxylysine, its picrate would contaminate the crude lysine salt isolated in this work.

Samples of crude lysine monopicrate obtained in analyses of the four protein preparations were mixed in approximately equal amounts and submitted to fractional recrystallization from aqueous solution. A disgusting odour, suggestive of mustard and CCl_4, was evolved when the mixture was dissolved in hot water. By repeated fractional recrystallization five fractions were obtained. The head fraction accounted for 85·1% of the weight of the mixture, successive fractions for 1·6, 3·4 and 3·3, and the tail fraction 6·1%; 0·5% was not recovered, and may have been lost in the effluvium referred to above.

The five fractions were submitted to so-called 'one-dimension' partition-chromatographic analysis (Consden, Gordon & Martin, 1944). The stationary phase was phenol-saturated water containing 0·3% (w/v) of NH_3, and the mobile phase was water-saturated phenol. Preliminary liberation and separation of the amino-acids from the picric acid was found to be unnecessary, a picrate (NH_4 salt?) band travelling independently of the amino-acids. From the intensities of the band colorations it was estimated roughly that the amino-acids in the original mixture of crude picrates probably contained 98% of lysine. Of the remainder, only a small fraction, if any, could have been hydroxylysine.

RESULTS

The results of the analytical work on the protein preparations are given in Tables 2 and 3. In Table 2 are shown the percentages of the leaf-protein nitrogen found in the form of arginine, histidine and lysine in the hydrolysates with assessments of the random errors of estimation. The table is self-explanatory.

Evaluation of the analyses

It seems likely that method 2 would have given, if applied generally, arginine values similar to those obtained by method 1, but with considerably smaller variability of replicate estimations (to judge from the work with the test mixtures). The relatively large variability of replicate arginine estimations by method 1 is attributable, at least in part, to the fact that, although the degree of purity of the isolated crude arginine monoflavianate must have varied,

Table 2. *Percentages* of protein* N *found in the hydrolysates in the forms of arginine, histidine and lysine (corrected only for systematic errors of analysis of hydrolysates), and other data*

Protein prep.†		N in prep.‡ (%)	Ash in prep.‡ (%)	Arginine N (%) Method 1	Arginine N (%) Method 2	Histidine N (%) Method 1	Histidine N (%) Method 2	Lysine N (%)
1 E	*Phalaris tuberosa*	14·90	1·8	12·5 (5)	—	2·10 (4)	3·58 (2)	6·60 (3)
2 E	*Hordeum murinum*	14·10	2·8	12·6 (4)	—	2·00 (4)	3·52 (3)	6·33 (4)
3 E	*Medicago sativa*	14·15	2·3	12·2 (2)	12·4 (2)	2·16 (2)	3·65 (3)	6·36 (2)
4 E	*Medicago denticulata*	14·55	1·4	11·6 (3)	—	2·00 (3)	3·86 (2)	6·51 (3)
'Within preparations' estimate of standard deviation				[0·42]	[0·14]	[0·229]	[0·034]	[0·303]

* In parentheses beside the values are indicated the numbers of estimations from which the values (the means) were derived.

† The serial cyphers are those assigned by Lugg & Weller (1944). ‡ Data from Lugg & Weller (1944).

Table 3. *Percentages* of the protein* N *occurring as arginine, histidine and lysine, the values having been corrected for hydrolysis losses believed to result from the presence of impurity*

Protein prep.		N in prep. (%)	Arginine N (%)	Histidine N (%)	Lysine N (%)
1 E	*Phalaris tuberosa*	14·90	13·7 (5)	3·66 (2)	6·60 (3)
2 E	*Hordeum murinum*	14·10	14·3 (4)	3·63 (3)	6·33 (4)
3 E	*Medicago sativa*	14·15	14·0 (4)	3·76 (3)	6·35 (2)
4 E	*Medicago denticulata*	14·55	13·0 (3)	3·97 (2)	6·51 (3)

Analysis of variance

Source of variation	Arginine D.F.	Arginine S.S.	Arginine Mean square	Histidine D.F.	Histidine S.S.	Histidine Mean square	Lysine D.F.	Lysine S.S.	Lysine Mean square
Between preparations	3	3·21	1·07	3	0·1521	0·0507	3	0·1526	0·0509
Within preparations	12	2·24	0·187	6	0·0070	0·0012	8	0·7325	0·0916
Variance ratio			5·7			42			0·56

As judged by the F variance ratio, there is a significant difference between the mean squares for arginine, a highly significant difference between those for histidine, and no significant difference between those for lysine (see discussion).

* In parentheses beside the values are indicated the numbers of estimations from which the values (the means) were derived.

a uniform correction factor was applied to the weights of crude salt in most of the analyses. It is clear that method 2 has yielded consistently larger histidine values than method 1.

Even if the correction for overall losses (+2·1 mg. N) were not applied, the values for histidine obtained by method 2 would still be much greater than the corrected values by method 1. Obviously, the histidine values obtained by method 1 cannot be accepted. The only explanation which seems to be in keeping with the careful examination, which Tristram (1939) made, is that, whereas at the stage of precipitation of the histidine diflavianate his test solutions probably contained very little amino-acid other than histidine, those derived from the protein hydrolysates probably contained rather more, and that the solubility of the histidine salt must be vastly increased in the presence of the other amino-acids. Our test mixtures contained large quantities of other amino-acids, but in any case Vickery & Winternitz (1944) have adduced reasons for believing that the solubility of histidine-DBS is probably only slightly affected by the presence of other amino-acids. Attention must be drawn to the unusually small variability of replicate histidine estimations made by method 2.

There is some uncertainty about the accuracy of the lysine values. Chibnall, Rees & Williams (1943) obtained good agreement between lysine values for β-lactoglobulin estimated by Tristram's (1939) method and by their own comprehensive method in which, likewise, lysine monopicrate is weighed. However, a comparison of these values with those obtained by isotope dilution, microbiological assay, specific decarboxylase, and electrodialysis (with N difference) methods, shows that the former are almost certainly too low (see Macpherson, 1946). Chibnall (1946) has given comparisons for some other proteins, the agreement being excellent in some instances and poor in others. In Tristram's (1939) method the initial precipitation of the lysine monopicrate occurs under such conditions (high concentration of ethanol) that the solubility of the salt is probably very low, whether other picrates are present or not. Our assessment of the purity of the crude lysine monopicrate was not based upon recrystallization losses. Consequently, if enhanced recrystallization losses constitute the major source of systematic error in such estimations, our values may be fairly reliable.

In compiling Table 3 we have returned to the individual values from which the mean values shown in Table 2 were derived. Of the histidine values only those obtained by method 2 have been used. All the arginine values have been used. Those for 3 E obtained by both methods have been grouped, as they differed but little among themselves, and the inclusion of the two values obtained by method 2 could not appreciably affect a general statistical analysis based upon the supposition that all values were obtained in the same way. The individual values have then been corrected on the following basis. The assumption has been made that during hydrolysis the protein preparations yielded all of these three amino-acids contained in them, that the destruction in the absence of carbohydrate or polyuronide impurities would not have been any greater than in the test 'hydrolysis' experiments (i.e. 0%), because the bulk of the amino-acids is probably liberated within the first several hours of hydrolysis (see Lugg, 1946), that the losses due to the presence of such impurity are proportional to the amount of it, that the amount can be calculated with sufficient precision from the known N contents of the preparations (corrected for ash) and the N contents (believed to be about 16·75%; see Lugg, 1939) of the hypothetical pure proteins, and that 1·00 g. of arabinose corresponds in quantity and effects with 0·88 g. of (pentosan) impurity (Lugg, 1939).

The impurities present in 2·5 g. quantities of the four preparations were thus calculated to have been equivalent in effects to the following quantities of arabinose: 0·27 g. (1E); 0·38 g. (2E); 0·39 g. (3E); 0·34 g. (4E). The corresponding corrections for the arginine and histidine values were calculated from the data in Table 1. On the basis of Tristram's (1939) work, no corrections were required for the lysine values.

Lugg (1939) was of the opinion that the common impurity in preparations like these may be pectin or more probably mucilage, rather than pentosan, and the corrections calculated on the above basis may require revision if, at some future time, recovery hydrolysis experiments are made with these other possible contaminants. The means of the corrected values are shown in Table 3. The corrected (individual) values were used in the analysis of variance given at the foot of the table.

DISCUSSION

Although an element of uncertainty must yet remain with respect to destruction of amino-acids during their hydrolytic liberation from proteins, recent extensive analyses of several 'pure' proteins (reviewed by Chibnall, 1946) suggest that the destruction, in most instances, is probably small. The results given in Table 3, in which corrections have been made for assessable losses, are to be viewed in the light of these remarks.

The arginine and lysine contents of the four protein preparations are of similar magnitudes to those found by Tristram (1939) for plant-leaf protein preparations which (for the most part) were probably less fully representative of the total protein in the leaves, but there is reason to believe that Tristram's (1939) arginine values may have been somewhat overestimated (see earlier remarks concerning methods and overall losses). The histidine contents of the four preparations are very considerably greater than those found by Tristram (1939) for the other preparations. It is to be noted, however, that when the method used by Tristram (1939) was applied to the four preparations, values similar to those he reported for the other preparations were obtained. For reasons already discussed, we have discarded the histidine values obtained by Tristram's (1939) method. We are of the opinion that the histidine values reported by Tristram (1939, 1940), and by Lugg (1943) who used the same method in an analysis of protein from the fronds of *Pteridium aquilinum*, were probably too low. Indeed, it seems likely, in the light of the discussion in the previous section, that almost all protein histidine contents obtained by the use of methods involving precipitation as diflavianate, whether they be large- or small-scale procedures, will require re-examination. This is implied also by the results of recent electrodialysis studies (Macpherson, 1946).

Attention has been drawn (Lugg & Weller, 1944) to the fact that the cyst(e)ine and methionine contents of the legume leaf proteins (3E, 4E) are appreciably lower than those of the grass leaf proteins (1E, 2E), and that the same tendency had been observed among protein preparations, many of which were probably far less fully representative of the total protein in the tissues. An interesting point arising from the present study is the astonishing uniformity in the arginine, histidine and lysine contents of the four preparations. Statistical examination of the values, with the aid of the analysis of variance given at the bottom of Table 3 and the *t* test, reveals that at the 95% probability level the differences in arginine values are significant only as between 4E and the other preparations, whereas the differences in histidine values, small though they are, are significant in all cases except as between 1E and 2E. However, the differences and their degrees of significance would be modified somewhat by the incidence of errors in the reported N contents of the protein preparations.

SUMMARY

1. The arginine, histidine and lysine contents of protein preparations, 1E, 2E, 3E and 4E, which are believed to be reasonably representative of the total protein in leaves respectively of *Phalaris tuberosa* L., *Hordeum murinum* L., *Medicago sativa* L., and *M. denticulata* Willd., have been estimated. As percentages of the protein N occurring in the forms of these three amino-acids, the values for the four plant species, respectively, may be expressed: arginine, 13·7, 14·3, 14·0, 13·0; histidine, 3·66, 3·63, 3·76, 3·97; lysine, 6·60, 6·33, 6·36, 6·51. The work involved an examination of methods of analyzing hydrolysates, and an examination of the probable effects of impurities in the protein preparations upon the analyses.

2. It is believed, on the evidence afforded by fractional recrystallization of amino-acid picrates and 'one-dimensional' partition-chromatographic analyses of the fractions, that the preparations contained no hydroxylysine.

We wish to thank Prof. M. H. Belz for assistance with the statistical examination of the data.

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Protein in Senescent Leaves of *Trifolium subterraneum*: Partial Amino-acid Composition

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It has been shown (Lugg & Weller, 1941) that in very young leaf tissue the whole protein composition may differ rather markedly from that of the whole protein in older tissue. (The axial organs of germinating seeds were used, but the bulk of the protein was present in the stalks and plumules.) In the following account it will be shown that at the other extreme of age (senescent tissue) the departure in composition of the whole-leaf protein from the pattern which seems to prevail during most of the life of the plant may again be fairly marked in some respects.

EXPERIMENTAL

Preparation of materials. The fresh plant material consisted of the leaves and attached petioles of *Trifolium subterraneum* harvested very late in the season (December, 1940) from senescent plants growing in a relatively cool and damp environment in the Adelaide foothills. 'Yellowing' was somewhat advanced in about a third of the leaf material, and much of the rest showed traces of it.

From the bulk of the fresh material an 'extracted' protein preparation (E) was made by the alkaline buffer lipid-solvent method (Lugg, 1939) in the manner described by Lugg & Weller (1944). 'Residual' (R) and 'whole' (W) protein preparations were also made by methods previously described (Lugg, 1939).

Data concerning the fresh material and the protein preparations made from it are shown in Table 1. The terms 'coagulable N' and 'protein N' have been explained fully in earlier publications (e.g. Lugg, 1939). For the rest, the table is self-explanatory.

Analytical work. Nitrogen estimations were made by the Kjeldahl method with Se catalyst. The amide, tyrosine, tryptophan, cyst(e)ine (i.e. cysteine plus cystine) and methionine contents of the protein preparations, W, E and R, were estimated by methods previously described (Lugg, 1937; 1938*a*, *b*, *c*; Lugg & Weller, 1941). The 'differential oxidation method' was used for the estimation of the cyst(e)ine and methionine contents. Considerable difficulty was encountered with the tryptophan estimations on the W and R preparations because of the unusually intense brown coloration of the alkali-stannite hydrolysates. Corrections for this coloration were so large that the precision of the estimations was seriously impaired.

The arginine, histidine and lysine contents of preparation E were estimated by methods recently described (Lugg & Weller, 1948) and based upon the procedures of Tristram (1939) and of Vickery & Winternitz (1944). These methods are considered to be unsatisfactory for use with preparations

Table 1. *Extraction of protein from senescent leaves of* Trifolium subterraneum

Fresh wt. taken (kg.)	Dry wt. (%)	N in dry leaf (%)			Extracted N (%)			Protein preparation	N in prep. (%)	Ash in prep. (%)
		Total	Coagulable	Protein	Total	Coagulable	Protein			
12·3	20	2·45	2·05	1·99	—	—	—	W	3·16	2·4
—	—	—	—	—	33	28	26	E	13·15	2·6
—	—	—	—	—	—	—	—	R	2·10	3·5

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like W and R. Of the protein N, there were estimated to be present in the hydrolysates, 11·2% by method 1 and 11·0% by method 2 (11·1% mean) as arginine, 4·07% by method 2 as histidine and 5·72% as lysine. In deriving this lysine value, weights of crude lysine monopicrate were corrected by multiplying them by the factor 0·975 determined previously for use in analyses of other leaf-protein preparations (Lugg & Weller, 1948). The arginine and histidine values required correction for losses attributable to the presence of interfering impurity in the protein preparation. The interfering impurity in 2·5 g. of preparation E was calculated from the N and ash contents of the preparation and assumed N content of 16·75% for the hypothetical pure protein (see Lugg & Weller, 1948) to correspond in quantity and effects with 0·55 g. of arabinose, and the above arginine and histidine values were accordingly multiplied by the appropriate factors (see Lugg & Weller, 1948).

RESULTS

The results are shown in Table 2, the partial compositions of the preparations being expressed as percentages of the protein N occurring in amide and the amino-acids concerned. Except for the tryptophan contents the random errors with the W and R preparations are believed not to have been more than 1·5 times as great as those with preparation E, and these in turn are believed not to have exceeded the following percentages (in parentheses) of the tabulated values: N (0·5, affecting all the other errors), amide (1·5), tyrosine (2), tryptophan (3), cyst(e)ine (4), methionine (5), cyst(e)ine plus methionine (2), arginine (4), histidine (2) and lysine (8). Estimates of the likely limits of random errors in the arginine, histidine and lysine values have been based partly upon the deviations encountered in the present work and partly upon those encountered in earlier work with these methods (Lugg & Weller, 1948). Those for the other values have been derived from much experience with the methods concerned.

Table 2. *Partial compositions of protein preparations from* Trifolium subterraneum

Preparation	N (%)	Percentages of protein N								
		Amide N	Tyrosine N	Tryptophan N	Cyst(e)ine N	Methionine N	Cyst(e)ine + methionine N	Arginine N	Histidine N	Lysine N
W	3·16	5·48	2·80	1·85	1·42	1·05	2·47	—	—	—
E	13·15	5·39	2·81	1·80	1·43	1·13	2·56	13·5	4·26	5·72
R	2·10	5·71	2·90	1·65	1·47	1·02	2·49	—	—	—

DISCUSSION

In view of the rather low protein content of the leaf material the yield of 'extracted' protein was not, for the procedure employed, unduly poor, nor was the purity of the preparation (judged from its N content) unexpectedly low. Presumably, therefore, no large fraction of the leaf protein was in a denatured condition. From a consideration of the amide, tyrosine, tryptophan, cyst(e)ine, methionine and cyst(e)ine plus methionine contents of the preparations, and of the rationale of the method of obtaining preparation E, it is believed that this preparation was reasonably representative of the whole protein in the leaf material.

Viewed broadly, the leaf protein composition appears to be similar to those found for other spermatophytes. Comparison of the contents of Table 2 may be made with those of tables in recent publications (Lugg & Weller, 1948; Lugg, 1946). A significant feature of these other tables is that the cyst(e)ine and methionine contents of the whole proteins in leaf tissue from reasonably mature plants are, in general, somewhat lower for the Leguminosae than they are for any of the other plant families investigated. For 'whole', 'extracted' and 'residual' protein preparations from *Trifolium subterraneum* specifically, Lugg (1943) found the cyst(e)ine-N values to be 1·15, 1·13 and 1·12%, respectively, and the methionine-N values to be 1·32, 1·38 and 1·29%, respectively, the cyst(e)ine-N plus methionine-N values being 2·47, 2·51 and 2·41%, respectively. The cyst(e)ine-N plus methionine-N values for preparations W, E and R of the present study are much the same as these, but it will be noticed that the cyst(e)ine contents are distinctly higher whilst the methionine contents are distinctly lower than those reported for the non-senescent material. Indeed, the cyst(e)ine contents are above the range of values, and (except for the seedling material examined by Lugg & Weller, 1941) the methionine contents below the range of values which have been estimated by this method for all other legume leaf materials. Again, the tyrosine values for preparations W, E and R, which are somewhat higher than those reported for the non-senescent material (Lugg, 1943), are among the highest yet encountered in leaf-protein preparations. The histidine content of preparation E is slightly higher than those of two legume- and two grass-leaf preparations recently examined (Lugg & Weller, 1948).

From past studies of plant-leaf protein preparations we have come to associate small, and usually rather uncertain, composition changes with different samples of leaf material harvested at different times during most of the life history of the plant. We do not know whether the protein in senescent tissue of

T. subterraneum would always correspond closely in composition with the amide and amino-acid values we have obtained in this one instance. In so far as it may be safe to generalize from the data at present available, the onset of senescence appears to have been accompanied by an increase in the cyst(e)ine content (and possibly in the tyrosine content) and a decrease in the methionine content of the whole protein in the leaf material. We do not know whether the compositions of individual proteins in the leaves have undergone change. It is possible that the only change has been in the proportions of the individual proteins. In this connexion it is pertinent to point out that change in haemoglobin composition with transition from foetal to adult mammalian life (as shown by Vickery (1944) for the histidine contents of foetal and adult bovine haemoglobins) may be due to a change in the ratio of two (or more) haemoglobin components (demonstrated for foetal and adult human material by Andersch, Wilson & Menten, 1944).

Effects of plant age and treatment (such as grazing) upon the cyst(e)ine and methionine contents of leaf proteins were judged by Smith & Wang (1941) to be fairly pronounced, but the validity of their conclusions was questioned by Lugg (1943) on the ground that the analytical methods they used would be highly unreliable with the impure leaf-protein preparations they analyzed. That changes in the cyst(e)ine and methionine contents of the whole protein in the leaves of various plants may occur, particularly at the extremes of plant age, seems very likely, but our earlier work, covering considerable ranges of the intermediate ages of plants, has revealed no definite, progressive changes.

SUMMARY

1. The amide, tyrosine, tryptophan, cyst(e)ine, methionine, arginine, histidine and lysine contents of protein preparations from senescent leaves of *Trifolium subterraneum*, expressed as percentages of the protein N occurring in these forms, were found to be 5·39–5·71, 2·80–2·90, 1·65–1·85 (best 1·80), 1·42–1·47, 1·02–1·13, 13·5, 4·26 and 5·72, respectively.

2. From comparison of these values with those previously found for non-senescent material (arginine, histidine and lysine values were not available) and from other considerations, it has been concluded tentatively that the onset of senescence was accompanied by an increase in the cyst(e)ine and possibly in the tyrosine content, and a decrease in the methionine content of the whole protein in the tissues.

We wish to thank Mr D. S. Riceman for providing the leaf material used in this work.

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Nicotinic Acid in Cereals

1. THE EFFECT OF GERMINATION

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Nutrition surveys have shown that under present conditions dietary supplies of nicotinic acid are being obtained largely from cereals. These vary widely in their nicotinic acid content. Maize and oats are poor sources, rice and barley are better and whole wheat is probably the best of the staple cereals.

Kodicek (1940) found that chemical methods gave higher results for maize than biological methods, and suggested that better agreement might be obtained if extraction were carried out with hot water instead of acid or alkali. Melnick (1942), reporting the results of a collaborative study, stated that with acid extraction chemical methods gave higher results than microbiological assays for low-extraction wheat flour. With aqueous extraction to exhaustion lower results were obtained by both methods, the chemical still being higher than the microbiological. James, Norris & Wokes (1947) confirmed Melnick's findings with acid extraction for low-extraction flour, and extended them to maize and yeast, using a modified chemical method which gave satisfactory agreement with microbiological assays for whole wheat, barley and other foods. Various American workers (Oser, Melnick & Siegel, 1941; Cheldelin & Williams, 1942; Krehl & Strong, 1944) have reported the presence in cereals of so-called precursors of nicotinic acid which, although water-soluble, are inactive microbiologically. Hydrolysis with acid or alkali, or enzyme treatment converts these 'precursors' into microbiologically active substances. Other American workers (Burkholder & McVeigh, 1942; Burkholder, 1943; Davis, Laufer & Saletan, 1943) have shown that the nicotinic acid content of wheat, barley, oats and maize increases after germination, thus indicating the occurrence of a precursor possibly related to the so-called precursor of the previous workers. We thought it desirable to study by chemical as well as by microbiological methods the day-to-day changes in nicotinic acid content during germination, and thus obtain a more complete picture.

METHODS

Chemical assays

These were made by the method described by James *et al.* (1947). The coefficient of variation of a single assay averaged 3·6 in 310 consecutive assays on 97 samples of cereals and cereal preparations.

Microbiological assays

The method of assay using *Lactobacillus arabinosus* 17/5 as test organism, first described by Snell & Wright (1941), was later modified by Krehl, Strong & Elvehjem (1943) and by Barton-Wright (1944). The latter method, or that described in a Report to the Society of Public Analysts (1946), was used in the present work. Results have been calculated by reference to the equation for the standard curve, calculated by the method of least squares, or by comparison of the slopes of the similarly calculated standard and sample curves. The latter method (Wood, 1945, 1946) is to be preferred, but valid assays will give closely agreeing results by either method. In some instances the limits of accuracy were calculated by the elegant, but somewhat lengthy, method of Finney (1945).

In 364 assays on 31 samples (see Table 1) the average coefficient of variation of a single assay lay between 6 and 7%.

Germination methods

Laboratory scale. Selected seeds (50–75 g.) were planted in sterile sand in Petri dishes, moistened with distilled water and grown in the dark in a warm room or at 27°. At the end of the germination period (which was varied in different experiments) the seeds were washed free of sand with distilled water, and the degree of germination was determined by observing in each seed the ratio of the length of the shoot (acrospire) to the length of the seed, as in the method of the Association of Official Agricultural Chemists (1945), but using an extended scale (i.e. 1–2, 2–4, above 4) to cover the greater amount of germination obtained. The seeds were sorted into groups according to this scale of acrospire ratios and the percentage in each group was calculated. The seeds were then heated at 70–80° overnight and the average weight per seed was determined for different degrees of germination by weighing the whole of the seeds in each group. The seeds were then ground to no. 60 powder and the moisture content determined by heating at 105° for 3 hr. This was used to determine the average dry weight per seed.

Large scale 'stocking method'. The seeds were placed in calico bags containing 6–7 lb. each. These were first steeped at 10–12° for 1–4 days, according to the material, and then placed on the germinating floor under the same conditions as in the normal malting of barley. Malting was allowed to proceed as usual, records being kept of temperature and moisture additions. Each day one of the bags was removed to the kiln to check enzyme action. The kilned seeds were then sent to King's Langley. Here, weeds and other foreign matter, of which there was not more than 1%, were removed by hand. The given batch was sampled and the samples were examined in the same manner as the laboratory-germinated samples to determine the degree of germination. The average dry weight per seed in this batch was determined by weighing representative samples of 50–100 seeds. At least 9 such samples were taken. The coefficient of variation of the mean, calculated from the individual results on these 9 samples, lay between 1 and 2 (see Table 6). Before assay, a portion of the batch was ground to no. 60 powder, care being taken to ensure that the whole of the material passed through the sieve and was thoroughly mixed. This powdered material was stored in air-tight bottles and used for assay as soon as possible. Malted cereals were found to keep better in the unground condition.

Preparation of malt extracts

Since kilning had checked enzyme action without destroying the enzymes, the action of the latter could continue during the preparation of malt extract. In regard to barley, evidence had already been obtained (Klatzkin, Norris & Wokes, 1946) showing that in the brewing of malt extract more riboflavin might be produced. We therefore thought it of interest to examine this possibility for nicotinic acid. The malt extracts were prepared by methods ensuring the maximum yield of protein and aneurin (see Organ, Duncan, Westbrook & Wokes, 1943), since this might also be expected to ensure high yields of other B vitamins.

RESULTS

Barley

Large-scale experiments

Six typical batches of English barley were examined before and after large-scale malting, and the malt extracts made from the respective malted barleys were also assayed. Mean results by the chemical method (see Table 2) showed no significant increases in nicotinic acid content at either stage.

Table 1. *Mean results by microbiological assay for the nicotinic acid content of samples*

B = Barley. *M* = Malted barley. *ME* = Malt extract.

Sample	Days germinated	No. of assays	Mean content (μg./g. dry wt.)
B 25	0	19	87 ±2·2*
M 25	12	15	103 ±1·9
ME 25	—	23	105 ±2·6
B 32	0	6	102 ±3·5
M 32	12	5	123 ±2·8
ME 32	—	6	114 ±2·2
B 35	0	19	109 ±2·2
M 35	12	23	117 ±2·5
ME 35	—	16	112 ±2·4
B 36	0	12	125 ±2·9
M 36	12	6	139 ±2·9
ME 36	—	6	122 ±1·4
B 38	0	15	115 ±2·7
M 38	12	19	121 ±3·0
ME 38	—	18	131 ±3·8
B 40	0	16	86 ±2·3
M 40	12	15	108 ±2·1
ME 40	—	19	128 ±2·7
Wheat	0	6	58·8±0·9
Wheat	10	6	62·8±0·8
Maize	0	19	31·4±0·4
Maize	1	6	34·6±0·7
Maize	2	4	36·8±0·8
Maize	3	5	31·9±1·1
Maize	6	4	27·3±0·75
Maize	9	4	26·6±0·45
Maize	12	16	33·8±0·4
Oats	0	8	11·8±0·27
Oats	4	6	17·0±0·33
Oats	8	8	19·8±0·56
Oats	12	14	21·0±0·28

* Standard error of mean.

Table 2. *Changes in nicotinic acid content of barley during malting and brewing, as shown by chemical assays*

Series no.	Nicotinic acid (μg./g. dry wt.) Barley	Malted barley	Malt extract	Increase during Malting (%)	Brewing (%)
25	99	104	106	5·1	2·0
32	111	116	116	4·5	0·0
35	123	112	125	−8·9	10·7
36	123	129	121	4·9	−6·5
38	127	126	122	−0·9	−3·2
40	112	120	122	7·1	0·9
Mean	116	118	119	1·9	0·4

Mean results by the microbiological method (see Table 1) showed an average increase of 14·7 % during malting, but no significant increase during the brewing of the malt extract. Each of the six samples showed, microbiologically, an increase during malting and in one sample this exceeded 25%. There was thus a divergence between the chemical and microbiological results. A comparison of these (Table 3) showed that, with malted barley and malt extract, the average results by the two methods were in good agreement, and the individual deviations were scarcely significant when compared with the respective coefficients of variation. On the other hand, with unmalted barley the mean chemical result was 12 % higher than the mean microbiological result. In five out of six samples the chemical method gave appreciably higher results, and in at least one of these (series 40) the difference was highly significant ($p = 0{\cdot}99$). Dr E. R. Dawson also kindly assayed this sample, barley 40, by the microbiological method and obtained a mean result of 90 μg./g. dry weight, confirming our microbiological result. With barley, therefore, the chemical method may give results appreciably higher than the microbiological method.

Table 3. *Comparison of chemical and microbiological results*

Series no.	Chemical results as % of microbiological results: Barley	Malted barley	Malt extract	Coefficient of variation of mean chemical result: Barley	Malted barley	Malt extract
25	114	101	101	4·5	0·03	1·2
32	109	94	102	7·4	7·8	4·9
35	113	91	112	4·6	3·6	4·9
36	98	93	99	2·0	4·6	4·1
38	110	104	93	3·9	3·5	5·1
40	130	111	97	1·6	5·9	5·3
Mean	112	99	101	—	—	—

These data summarize results obtained in 170 assays. At least 4 assays were made on each sample.

The results of microbiological assays indicate an apparent increase in nicotinic acid content when barley is malted. However, seeds lose weight on germination—the so-called malting loss—and this must be taken into account in assessing the true change in vitamin content. We therefore determined the average dry weight of a barley seed before and after malting. The results showed an average loss of 12–13 % in weight, sufficient to account for almost the whole of the observed increase in nicotinic acid content. Hence, any nicotinic acid which had been formed from a precursor must have been used up in metabolic processes.

Small-scale experiments

In these, the barley was kept at 27° for 10 days, a sample from the material which was malted on the large scale being used. Acrospire counts showed that in some of the barley the degree of germination was considerably higher than in normal malting, the shoot being longer than the seed in 33 % of the seeds, as compared with less than 1 % in normal malting. Nevertheless, no significant increases in nicotinic acid content were obtained.

Wheat

A large-scale experiment was carried out in which 1 cwt. of English wheat was germinated at 14–20° for 12 days. Perhaps owing to the warm weather, there was some development of mould after 10 days on the floor. The experiment was discontinued at that point, when the chemical results (Table 4) showed a significant increase of 33 % in the nicotinic acid content, after allowance for the loss in weight of the seeds. Microbiological assays on the initial and final samples showed no increase, the average content of a single seed being 2·30 μg. before germination and 2·27 μg. after germination (both calculated on dry weight). On the ungerminated wheat the chemical result was 108 % of the microbiological. On the germinated wheat the chemical result was 147 % of the microbiological result, a significant difference.

Table 4. *Changes in dry weight and nicotinic acid content during germination of wheat*

Stage of malting		Nicotinic acid content by chemical method						
		Individual assays (μg./g. dry wt.)					(μg./seed)	
Days steeping	Days on floor					Mean	Actual	As % of initial
0	0	64·5	56·6	65·7	65·6	63·8	2·46	100
2	6	68·2	67·5	—	—	67·9	2·46	100
2	9	85·3	84·9	83·5	82·9	84·2	2·82	115
2	10	92·3	91·6	—	—	92·0	3·24	132

Maize

A large-scale experiment was carried out with 1 cwt. of maize (White Horse Tooth variety) germinated at 15–18° for 12 days. Acrospire counts and weight changes showed that a fair degree of germination had occurred, but there was no increase in nicotinic acid content when allowance was made for changes in dry weight. The chemical method showed a net loss of 6 % after 1 day on the germinating floor, and of 10 % after 12 days on the floor. The microbiological method showed no significant change. The chemical results were 107–116 % of the microbiological results.

A small-scale experiment was carried out on the same sample in a warm room at 16–22° and also showed no increase in nicotinic acid content, although the acrospire counts indicated that a considerable degree of germination had occurred, the percentages of seeds with different acrospire ratios being as follows: 0–0·5, 9 %; 0·5–1, 56 %; 1–2, 15 %; 2–4, 13 %; above 4, 7 %. After 8 days' germination there was a net loss of about 12 %, and after 10 days a net loss of about 15 % of nicotinic acid.

Rice

For this, we used paddy containing the husk. The small quantity available permitted of only small-scale experiments at 27°. In 9 days' germination the average net increase in nicotinic acid content was 17 % (Table 5). Of the seeds 93 % had germinated, but germination was rather uneven. Of the germinated seeds, 52 % had an acrospire ratio greater than 1·0. There was a much greater increase in nicotinic acid content in the seeds showing the greater degree of germination. In 7 days' germination 84 % of the seeds had germinated and the increase in

Table 5. *Changes in dry weight and in nicotinic acid content during germination of rice (paddy) as shown by chemical assays*

Period of germination (days)	Acrospire ratio	Nicotinic acid content				
		Individual assays (μg./g. dry wt.)			(μg./seed)	
				Mean	Actual	As % of initial
0	—	74·1	73·0	73·6	1·88	100
7	1–4 (12 %)	95·2	100·0	97·6	2·41	128
9	Below 1 (48 %)	79·3	82·4	80·9	1·85	98
9	1–2 (33 %)	96·0	95·3	95·7	2·30	122
9	Above 2 (19 %)	124·0	116·4	120·2	2·85	152

Figures in parentheses represent percentage of total quantity of germinated rice which had reached the stated degree of germination.

seeds with acrospire ratios above 1·0 was 28 % as compared with 33 % for the corresponding degree of germination after 9 days. So far as we are aware, this is the first time that a significant increase in nicotinic acid content has been shown to occur in rice as the result of germination. It would seem worth while to carry out experiments on a larger scale when more material becomes available.

Oats

A large-scale experiment was carried out in which 1 cwt. of English oats was germinated at temperatures ranging from 15 to 18° for 12 days. The results (Table 6) showed by both chemical and microbiological methods almost the same percentage increase above the respective initial values. The average nicotinic acid content/seed by the microbiological method was, however, only about two-thirds of that determined chemically for the same sample. The differences between the chemical and microbiological figures were all highly significant ($p=0·99$). Each of the chemical figures in Table 6 is the mean obtained from at least four assays. The coefficients of variation of the mean (as calculated from the respective standard deviations of the mean) for the figures for samples 1–13 ranged from 0·2 to 3·7 and averaged 1·4. For sample 14 there was a higher coefficient of variation of 8·5 obtained from seven assays. We cannot explain why the results for this sample showed this wider deviation. However, the nicotinic acid content/seed for sample 14 falls into line with the nicotinic acid content found in the other samples. The rate of increase in nicotinic acid content of an average seed in the latter part of the experiment had slowed down considerably since the rapid initial increase.

Table 6. *Changes in nicotinic acid content and average weight/seed during germination of oats*

			Nicotinic acid content				
			Chemical method			Microbiological method	
				(μg./seed)		(μg./seed)	
Sample no.	Treatment	Average dry weight of a seed (mg.)	(μg./g. dry wt.)	Actual	As % of initial	Actual	As % of initial
1	Untreated	35·3±0·41*	19·5	0·69	100 ±0·3*	0·42	100
2	Steeped 24 hr.	32·0±0·79	23·0	0·74	107 ±3·2	—	—
3	Steeped +1 day on floor	32·8±0·54	26·9	0·88	127·5±0·6	—	—
4	Steeped +2 days on floor	35·7±0·50	27·2	0·97	141 ±5·2	—	—
5	Steeped +3 days on floor	32·9±0·31	30·7	1·01	146 ±0·4	—	—
6	Steeped +4 days on floor	29·5±0·37	28·3	0·83	123 ±0·2	0·50	119
8	Steeped +6 days on floor	32·1±0·28	27·8	0·89	129 ±1·7	—	—
10	Steeped +8 days on floor	30·2±0·47	32·8	0·99	143 ±1·1	0·60	143
12	Steeped +10 days on floor	30·7±0·54	34·9	1·07	155 ±3·1	—	—
13	Steeped +11 days on floor	30·8±0·44	30·8	0·95	138 ±1·7	—	—
14	Steeped +12 days on floor	31·5±0·64	34·4	1·08	157 ±13·4	0·66	157

* Standard error of mean.

The nicotinic acid content for each sample is the mean of at least 4 assays, of which the extreme range in the initial sample was 98–104 and in the final sample 92–108 % of the respective mean.

Acrospire counts on the samples in the large-scale experiment showed only a moderate degree of germination. We therefore carried out on the same batch of oats a small-scale experiment, aiming at a higher degree of germination by using a temperature of 27°. Acrospire counts showed that this had in fact occurred. After 10 days' germination, the chemical method showed a net increase of 108 % compared with 43 % in the large-scale experiment.

DISCUSSION

American workers using microbiological methods first showed that germination of cereals may, under certain conditions, increase their nicotinic acid content. Our own experience leads us to suggest that in further investigations it would be desirable to relate any observed increases to the degree of germination, as determined from acrospire counts and weight changes. This might explain some of the discrepancies between the findings of different workers and also help to build up a more complete picture of the germination process. For barley, e.g., Burkholder (1943) obtained with a single sample an increase of 70 % in nicotinic acid content/g. dry weight. When allowance was made for loss in weight during germination about one third of this apparent increase disappeared. We did not obtain a significant net increase with any one of our six samples in assays either by chemical or by microbiological methods. Moreover, there was still no significant increase when we prepared malt extracts from the malted barleys and thus gave still further scope to the germination enzymes. Our findings agree with those of Davis *et al.* (1943) who obtained an average increase of about 11 % in seven samples of barley as the result of germination, but made no allowance for loss in weight during germination, which would probably explain the whole of their observed increase.

With wheat, Davis *et al.* (1943) obtained an average increase of about 15 % in five samples. Our results indicate that the loss in weight during germination of wheat seeds (Fig. 1) might easily reach 15 %. If allowance were made for this loss in weight, the results of Davis *et al.* would probably not show any significant increase. Burkholder (1943) obtained a net increase of 24 % with a single sample. We obtained a net increase of 33 % by the chemical method, but no increase microbiologically.

Maize is of particular interest because of its low initial content of nicotinic acid, which has been held responsible for the prevalence of pellagra in parts of the world where it is a staple food. Burkholder & McVeigh (1942) obtained an increase of 141 %, uncorrected for loss in weight during germination. Burkholder (1943), after making such correction, obtained a net increase of 258 % on another sample. We did not obtain, either by chemical or microbiological methods, any increase in a sample with an initial content of 35·9 μg./g. dry matter. This may have been due to the high initial content as compared with 17 and 9·5 μg./g. dry matter respectively in the samples of the American workers. American findings indicate that greater increases may be expected with cereals of lower initial nicotinic acid content. Our results, on the whole, support this suggestion, but rice was an exception, since, using a high degree of germination, we secured an increase of 52 % in a sample with an initial content of 74 μg./g. The mean increase in the whole sample after 9 days' germination was only 17 %. In the work above our chemical assays had given the same general picture as our microbiological assays, and thus provided additional evidence of the reliability of the chemical method (cf. James *et. al.* 1947).

Oats, however, told a different story. Our chemical results for oats ungerminated and at different stages of germination were consistently 60–70 % higher than the microbiological values, though both methods showed the same net increase (57 % of the respective initial values) as the result of germination. This increase was considerably lower than the net increase of 352 % obtained by Burkholder (1943) microbiologically with one sample of oats. He, however, used oats with a much lower initial value and also obtained a much higher degree of germination. Fig. 1 shows the loss in weight at different stages of germination in our various samples, the data being used to calculate the net changes in nicotinic acid content recorded in our tables. The discrepancy between the chemical and the microbiological results was clearly established by the results of numerous assays, and will require further investigation.

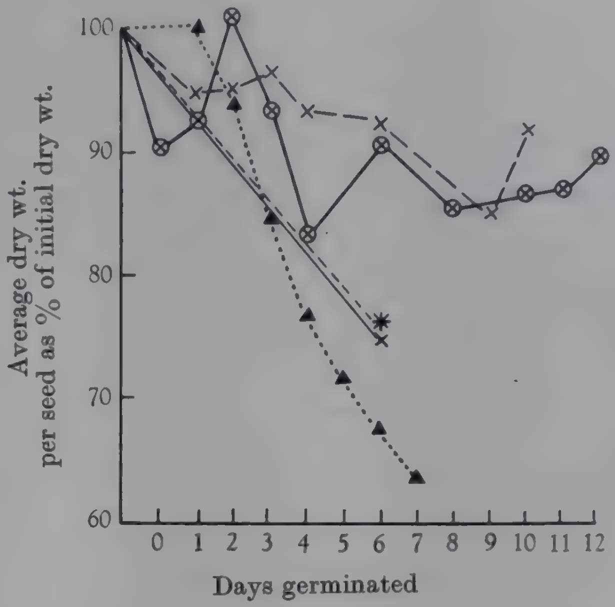

Fig. 1. Changes in dry weight of seeds during germination. All seeds in our experiments were steeped in cold water for 24 hr. before germination. Results obtained on wheat by Burkholder (1943) ×——×, Klatzkin, Norris & Wokes (present study) ×— —×; on oats by Burkholder (1943) ✳- - - -✳, McVeigh (1944) ▲· · · ·▲, Klatzkin, Norris & Wokes (present study) ⊗——⊗.

SUMMARY

1. Samples of barley, maize, rice (paddy) and oats have been germinated in the laboratory under known conditions. Large-scale germination experiments have been carried out on samples of barley, wheat, maize and oats. The degree of germination was followed by acrospire counts and by changes in dry weight. Nicotinic acid assays have been made on the dried powdered material, both by a chemical and a microbiological method, at different stages of germination.

2. In oats a net increase was obtained of 57–108 %. Rice gave net increases of 0–52 % with different degrees of germination. With wheat, barley and maize no significant increase was obtained.

3. It is suggested that the considerably greater increases reported by American workers may be due to their obtaining a higher degree of germination, and to some extent also to their omitting to allow for changes in weight during germination.

4. The chemical method gave satisfactory agreement with the microbiological method with all samples, except the wheat and oats and one sample of unmalted barley, where the chemical method gave higher results.

We are indebted to the Curator of the Imperial Institute, South Kensington, for a sample of Burma paddy, to Samuel Thompson and Sons Ltd. for carrying out large-scale malting of our samples of barley, wheat, oats and maize, and to Miss J. Horsford and Mrs R. Wallach for assistance. The work undertaken by one of us (C.K.) in this investigation forms part of the work for a Ph.D. thesis in the University of London.

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A Polarographic Study of some Stilbene Derivatives of Biological Interest

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4-Aminostilbene and some related compounds have been shown to have an inhibitory action on the growth of young rats and on malignant tumours (Haddow, Kon & Harris, 1945). Certain of these substances are also potent carcinogens capable of eliciting tumours at sites remote from that of administration (Haddow, Kon & Harris, 1946). The ethylenic bond present in stilbene has been found to be electroreducible at the dropping mercury electrode (Laitinen & Wawzonek, 1942) and the present investigation was undertaken for the purpose of comparing the polarographic behaviour of compounds of this series showing differing biological activities. It was also considered that the data obtained in such experiments would be of value in assessing the suitability of the polarographic method of analysis in metabolic studies. Since stilboestrol is also a stilbene derivative (4:4′-dihydroxy-$\alpha\beta$-diethylstilbene), and similar problems arise in connexion with investigations of its metabolism in the human subject (Warren, Goulden & Robinson, 1948), it was decided to include synthetic oestrogens of this type in the investigation.

EXPERIMENTAL

Materials

All the substances used in these experiments were pure compounds. A number of the stilbene derivatives containing basic substituents was kindly put at our disposal by Prof. G. A. R. Kon and Dr R. J. C. Harris. We are also greatly indebted to Prof. E. C. Dodds and Dr W. Lawson for generous gifts of stilboestrol derivatives.

Solvent and electrolyte

The solvent employed was a dioxan-water mixture (3 : 1 by vol.) (Laitinen & Wawzonek, 1942). Commercial dioxan (British Drug Houses Ltd. 'purified') was refluxed for 12 hr. with sodium and then fractionated. Repetition of the sodium treatment was sometimes necessary in order to achieve very negative decomposition potentials. Only recently purified dioxan was used since there was some deterioration on storage.

Laitinen & Wawzonek recommend the use of tetrabutylammonium iodide as a supporting electrolyte for the attainment of very negative discharge potentials. In the present experiments tetraethylammonium iodide was found to be quite suitable, and it was easily prepared by heating together triethylamine and ethyl iodide in absolute ethanol on a steam bath. The quaternary salt is very sparingly soluble in ethanol and rapidly separates. The salt was collected and recrystallized from absolute ethanol. It is stable and not deliquescent. The base solution was a 0·1 M-solution of this electrolyte.

Polarograms

The current-voltage curves were recorded on a Tinsley ink-recording polarograph. Control curves were first recorded using the base solution alone. The curve for the base solution containing the substance under examination was then superimposed on the record of the control curve. In all the measurements the observed diffusion currents were corrected for the residual current given by the base solution. O_2 was removed from the solutions by flushing with a current of H_2. The anode was a Hg pool of relatively large area and its potential was measured against a saturated calomel electrode (S.C.E.). The anodic potential was reproducible at −0·41 V. All cathodic potentials are also referred to the S.C.E.

The dropping Hg electrode had the following characteristics. At a pressure of 71·0 cm. of Hg the drop time (t) in the electrolyte solution used was 3·05 sec. (open circuit). The Hg flow (m) was 1·96 mg./sec. The calculated value of $m^{\frac{2}{3}} t^{\frac{1}{6}}$ is 1·887. The value of m was determined by direct weighing up to a negative potential of 2·4 V., and was found to be constant over this range. Direct measurements of t were also made over the same range, but the short drop times occurring at potentials more negative than 2·0 V. were checked independently by measurements of the diffusion current of nitrobenzene over the range −1·4 to −2·4 V. The effect of the decreased drop time reduced the product $m^{\frac{2}{3}} t^{\frac{1}{6}}$ from a value of 1·837 at −1·4 V. ($t = 2·6$) to 1·566 at −2·4 V. ($t = 1·0$), and the observed diffusion current of nitrobenzene showed a similar linear decrease over this range. All measurements were carried out at 25°.

RESULTS

In Table 1 the results obtained with stilbene derivatives not bearing substituents on the ethylenic carbon atoms are shown. Table 2 contains the measurements on compounds containing substituents in these positions. For each compound the range of concentration over which measurements were made is given together with the half-wave potential $E_{\frac{1}{2}}$ (*v.* S.C.E.), and the observed diffusion current i_d, after correction for the residual current. The half-wave potentials (in the tables negative signs have been omitted) have not been corrected for iR drop; with the type of cell used this correction would be small. The diffusion current shown by a mM-solution has been calculated from the measurements made at each concentration. The essential constancy of the half-wave potential with varying concentration of the substance, in those compounds that permitted its measurement, indicates that the electrode process is independent of the concentration of hydrogen ions, and justifies the use of unbuffered solutions in these measurements.

DISCUSSION

Two of the compounds included in this investigation have been examined by other workers (Laitinen & Wawzonek, 1942). For stilbene they reported $E_{\frac{1}{2}} = -2·14$ V. (*v.* S.C.E.) and $i_d/C = 4·04$. The corresponding values for this substance from Table 1 are −2·17 and 4·12. Although the half-wave potential determined in the present experiments agrees with that found by Laitinen & Wawzonek, the agreement between the values for i_d/C is less good when allowance is made for the difference in capillary characteristics. At the voltage used by Laitinen & Wawzonek for measurement of the diffusion current of stilbene (−2·31 V.), $m^{\frac{2}{3}} t^{\frac{1}{6}}$ had a value of 1·828 in their experiments, while the corresponding value in the present experiments was 1·593. If correction is made for the difference between the two factors the value of i_d/C shown in Table 1 becomes 4·73 as compared with the value of 4·04 given by Laitinen & Wawzonek.

The same workers also measured triphenylethylene and their values of $E_{\frac{1}{2}} = -2·12$ and $i_d/C = 3·55$ may be compared with the data in Table 2. Again agreement between values for the half-wave potentials is good, but the value for i_d/C found in the present experiments is higher (3·86) and, when corrected for difference in capillary, considerably higher (4·31) than that found by the American workers.

The half-wave potentials of most of the compounds shown in Table 1 fall within a narrow range. In the stilbene series, substitution in one or both nuclei results in a slightly more negative half-wave potential. The substituted stilbene analogues containing a naphthalene nucleus (last four compounds in Table 1), however, give waves at potentials equal to that of stilbene or more positive. This is particularly striking in the case of α-4-dimethylaminophenyl-β-2′-naphthylethylene. The second wave shown by these substances at approximately −2·50 V. is presumably due to reduction of the naphthalene nucleus, since reduction of this hydrocarbon gives a wave at this potential.

The general similarity of the values of i_d/C for most of the compounds in Table 1 indicates that there are no large differences in diffusion coefficients among these substances. In the cases of 4-amino-4′-hydroxystilbene and 4-amino-4′-hydroxy-2′-methylstilbene, which show rather lower values for i_d/C, it seems probable that these are due to changes in the nature of the potential-determining reaction at the dropping electrode rather than to reductions in diffusion rates. From the recorded current-voltage curves, plots were made of log $i/(i_d - i)$ against voltage. In general the reciprocal slopes of these graphs were in approximate agreement with the theoretical value of 0·059 V.

for a reversible, potential-determining reaction involving one electron. However, the plots of the data for 4-amino-4′-hydroxystilbene and for 4-amino-4′-hydroxy-2′-methylstilbene gave slopes of 0·11 V; Although the nature of the available data precluded a high degree of accuracy in plotting these derived curves, there seems little doubt that the electrode process differed in these compounds. Stilbene derivatives bearing both amino and hydroxyl groups in the 4 and 4′ positions are very susceptible to oxidation and it is possible that small quantities of such oxidation products were present in the specimens used.

Table 1. *Polarographic data for stilbene derivatives not bearing substituents on the ethylenic carbon atoms*

Compound	$-E_{\frac{1}{2}}$ (V. *v.* S.C.E.)	i_d (μamp.)	C (mM)	i_d/C
Stilbene	2·17	2·70	0·655	4·12
	2·17	5·40	1·310	4·12
4-Aminostilbene	2·30	2·12	0·646	3·28
	2·32	4·42	1·292	3·42
	2·31	6·68	1·938	3·44
4-Methoxystilbene	2·24	1·48	0·398	3·72
	2·24	3·04	0·796	3·82
	2·25	4·39	1·194	3·78
4-Hydroxystilbene	2·34	1·50	0·476	3·15
	2·34	2·27	0·714	3·18
	2·34	2·94	0·952	3·09
4-Dimethylaminostilbene	2·29	2·18	0·588	3·71
	2·29	4·39	1·176	3·73
	2·29	6·41	1·764	3·64
3-Dimethylaminostilbene	2·19	1·62	0·453	3·57
	2·19	3·29	0·906	3·63
	2·19	4·81	1·359	3·54
4-Amino-4′-hydroxystilbene	2·45	1·03	0·446	2·31
	2·44	2·00	0·892	2·24
	2·46	3·02	1·338	2·26
4:4′-Dimethoxystilbene	2·33	1·19	0·320	3·72
	2·31	2·34	0·640	3·66
	2·32	3·53	0·960	3·68
4-Dimethylamino-2′-methylstilbene	2·32	1·76	0·518	3·40
	2·30	3·60	1·036	3·48
	2·30	5·49	1·554	3·53
4-Dimethylamino-3′-methylstilbene	2·34	1·60	0·554	2·89
	2·33	3·68	1·108	3·32
	2·33	5·56	1·662	3·34
4-Dimethylamino-4′-methylstilbene	2·36	1·87	0·565	3·31
	2·35	3·67	1·130	3·25
	2·34	5·70	1·695	3·36
4-Dimethylamino-2′:5′-diethylstilbene	2·37	0·90	0·303	2·97
	2·33	1·90	0·606	3·14
	2·34	4·43	1·515	2·92
4-Amino-4′-hydroxy-2′-methylstilbene	2·41	1·74	0·724	2·40
	2·44	2·49	1·086	2·30
2:4:6:2′:4′:6′-Hexamethylstilbene	2·47	1·08	0·752	1·44
	2·48	2·48	1·880	1·32
α-4-Dimethylaminophenyl-β-1′-naphthylethylene	2·12 2·50	1·32	0·322	4·10
	2·12 2·50	2·70	0·644	4·19
	2·10 2·50	3·94	0·966	4·08
α-4-Dimethylaminophenyl-β-2′-naphthylethylene	1·80 2·49	0·87	0·188	4·63
	1·82 2·49	1·90	0·470	4·06
α-4-Dimethylaminophenyl-β-1′-(2′-methyl)-naphthylethylene	2·18	0·73	0·239	3·05
	2·17	1·60	0·478	3·34
	2·17 2·54	3·75	1·195	3·14
α-Phenyl-β-1-(4-dimethylamino)naphthylethylene	2·08 2·53	—	—	—

Certain of the compounds in Table 1 are of especial interest since they possess carcinogenic properties. 4-Aminostilbene, 4-dimethylaminostilbene, 4-dimethylamino-2′-methylstilbene and α-4-dimethylaminophenyl-β-1′-naphthylethylene have all been reported as inducing malignant tumours in rats (Haddow *et al.* 1945, 1946). From the data of Table 1 there is nothing to distinguish such biologically active substances from an inactive one such as stilbene.

Table 2. *Polarographic data for stilbene derivatives bearing substituents on the ethylenic carbon atoms*

Compound	$-E_{\frac{1}{2}}$ (V. *v.* S.C.E.)	i_d (μamp.)	C (mM)	$-i_d/C$
α-Ethylstilbene	2·30	1·57	0·408	3·84
	2·31	3·14	0·816	3·84
	2·31	4·65	1·224	3·80
αβ-Diethylstilbene (m.p. 71·5–72·5°)	*NW*	—	—	—
αβ-Diethylstilbene (m.p. 74–75°)	(2·59)	—	—	—
Stilboestrol (4:4′-dihydroxy-αβ-diethylstilbene)	*NW*	—	—	—
Dienoestrol (3:4-di-(4′-hydroxyphenyl)hexa-2:4-diene)	*NW*	—	—	—
4:4′-Dihydroxy-α-methyl-β-ethylstilbene	*NW*	—	—	—
4:4′-Dihydroxy-α-methyl-β-cetylstilbene	*NW*	—	—	—
4:4′-Dihydroxy-α-phenyl-β-ethylstilbene	*NW*	—	—	—
2:2′-Dihydroxy-αβ-diethylstilbene	*NW*	—	—	—
4-Methoxy-αβ-diethylstilbene	*NW*	—	—	—
4:4′-Diacetyl-αβ-diethylstilbene	1·77	1·34	0·172	7·80
	1·78	2·71	0·345	7·85
	1·79	4·07	0·517	7·85
4:4′-Dihydroxy-α-*n*-propylstilbene	2·52	2·80	1·890	1·47
4:4′-Dihydroxy-α-*n*-amylstilbene	2·53	1·80	1·570	1·15
4:4′-Dihydroxy-α-*iso*propylstilbene	(2·61)	—	—	—
Triphenylethylene	2·12	1·22	0·328	3·72
	2·09	2·53	0·656	3·86
	2·10	3·80	0·984	3·86
Triphenylmethylethylene	2·24	1·20	0·330	3·64
	2·25	2·45	0·660	3·72
	2·24	3·59	0·990	3·63
α-4-Hydroxyphenylstilbene	2·17	0·86	0·266	3·23
	2·17	1·75	0·532	3·29
	2·17	2·68	0·798	3·35
α-Phenyl-ββ-di-4-hydroxyphenylethylene	2·28	1·04	0·320	3·25
	2·29	2·28	0·640	3·56
α-Ethyl-β-4-hydroxyphenylstilbene	2·36	2·20	0·467	4·72

Compounds showing no reduction at the dropping electrode are indicated by *NW* (no wave). Two compounds appeared to give indications of waves at very negative potentials and these values are included in parentheses.

It is interesting to compare the data for these substances with those for other carcinogens. Wawzonek & Laitinen (1942) showed that, although aromatic compounds possessing a high degree of resonance, such as benzene, are not reduced at the dropping mercury electrode, aromatic polynuclear hydrocarbons showing a diminished degree of resonance are reducible. In experiments which have been confirmed and extended by the present authors, they established the polarographic behaviour of such carcinogens as 3:4-benzpyrene, methylcholanthrene, 1:2:5:6-dibenzanthracene, and 1:2-benzanthracene. The polarographic waves resulting from reduction of these compounds in aqueous dioxan were compared with the waves developed by anthracene, naphthalene, and phenanthrene under the same conditions.

In general, the polynuclear aromatic hydrocarbon carcinogens give two waves at approximately −1·90 and −2·50 V. These waves are characteristic for the reduction of an anthracenic structure (9 : 10 addition) followed by that of the resulting naphthalenic nucleus (1 : 4 addition). The polarographic reduction resembled, in the relative ease and site of attack, such reactions as the addition of alkali metals or reduction by ethanol and alkali metals.

From the data in Table 1 it is clear that the susceptibility of the ethylenic bond of stilbene derivatives to reactions involving addition at this site is intermediate between that shown by the anthracenic and naphthalenic bonds of the hydrocarbon carcinogens. Possibly, with stilbene derivatives, as with polycyclic aromatic hydrocarbons, this degree of susceptibility is an important feature in their adsorption on to enzymes. From a comparative point of view the stilbene ethylenic bond is analogous to the 1 : 2 double bond in pyrene ($E_{\frac{1}{2}} = -2{\cdot}10$) rather than to the 9 : 10 double bond in phenanthrene ($E_{\frac{1}{2}} = -2{\cdot}44$).

All but one of the compounds shown in Table 2 are α- or αβ-substituted stilbene derivatives. The exception is dienoestrol in which there is a conjugated hexadiene system. In general, monosubstitution on the α-carbon atom results in reducible derivatives with half-wave potentials approximately the same or slightly higher than that of stilbene itself. This applies to both α-alkyl and α-aryl derivatives. The nature of the substituent groups (alkyl or aryl) is, however, of decisive importance in disubstituted derivatives. Stilbene derivatives with alkyl groups on both α and β carbon atoms are, in general, not reducible at the mercury cathode. In the exceptional case of αβ-diethylstilbene, two specimens were available; one had m.p. 71·5–72·5°, the other m.p. 74–75°. These are probably the *trans* and *cis* forms, respectively, of this hydrocarbon (Goulden, unpublished). Although the constitution of these two substances has not yet been finally established, the evidence at present available suggests that the *trans* form is not reducible. The higher-melting form gave some evidence of a very ill-defined wave at approximately −2·6 V. Apart from this one rather doubtful exception, αβ-dialkyl derivatives of the stilbene and stilboestrol series were not reducible. The large wave shown by 4:4′-diacetyl-αβ-diethylstilbene is presumably produced by reduction of the keto groups, since reduction of acetophenone under the same conditions gave a wave at about the same potential (−1·67 V.). Disubstitution with one alkyl and one aryl group produces reducible derivatives, e.g. triphenylmethylethylene and α-ethyl-β-(4-hydroxyphenyl)-stilbene.

One object of the present work was to explore the possibility of using polarographic methods of analysis in the detection and estimation of stilbene derivatives in metabolic experiments. The results indicate at least one serious practical difficulty. The half-wave potentials lie between about −2·2 and −2·6 V., i.e. more negative than the discharge potentials of ions of the alkali metals or of ammonium (*c.* −2·2 V.). The presence of small amounts of these ions makes it impossible to detect or measure the reduction of stilbene derivatives. Preliminary experiments on blood and urine from rats treated with 4-dimethylaminostilbene showed that the preparation of extracts suitable for polarographic measurements (i.e. free from Na^+, K^+ and NH_4^+) was difficult, and in no case was a satisfactory measurement obtained.

SUMMARY

1. Polarographic data are presented on 37 compounds of the stilbene and stilboestrol series.

2. Although certain of the stilbene derivatives possessed carcinogenic potency, no differentiation could be made between these compounds and related non-carcinogenic stilbenes.

3. Stilbene derivatives of the types, α-alkyl, α-aryl, or α-alkyl-β-aryl, are reducible at the dropping mercury electrode. Derivatives of the type αβ-dialkyl are not reducible.

4. αβ-Dialkylstilboestrols and dienoestrol are not reducible.

5. The highly negative half-wave potentials of stilbene derivatives make the use of polarographic methods difficult for the detection and estimation of these substances in biological materials.

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An Electrophoretic Study of Diphtheria Toxoid Preparations

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It has been found that batches of diphtheria toxoid produced in these laboratories for the immunization of horses exhibit varying degrees of antigenicity. This variation has not been correlated with any variations in the constitution of the medium used for the toxin production or in the conditions of growth of the organism thereon. Since it would be of considerable advantage to possess a method of rapidly assessing the probable antigenic quality of a toxoid preparation prior to its injection into horses, it was thought that an investigation of the electrophoretic behaviour of different batches of toxoid would be worth while, as providing a possible criterion of antigenic potentialities.

Although the results of these investigations did not reveal any consistent differences among groups of toxoid preparations which were known to produce good and poor antigenic responses, a number of points arose which are worth recording. A considerable amount of work has been carried out on the electrophoretic behaviour of the corresponding antibodies (cf. Kekwick & Record, 1941), but data referring to diphtheria antigens are confined to observations by Lundgren, Pappenheimer & Williams (1939), who published a short account of the electrophoretic behaviour of highly purified diphtheria toxin; they did not extend their work to toxoid.

EXPERIMENTAL

Production and preparation of toxoids. Most of the toxoids were prepared from toxins produced on a papain digest of beef medium, except for one produced on a tryptic digest of beef, and another on an acid hydrolysate of casein medium. These preparations had to be concentrated 40–50 times to provide a solution strong enough for the investigation. Concentration was carried out by means of an ultrafilter, and, as dialysis against buffer caused some loss of toxoid through the membrane, the toxoid was thoroughly washed on the ultrafilter with the requisite buffer solution until the refractive indices of the washings and of fresh buffer were indistinguishable within the limits of error of the dipping refractometer. The refractive index of the toxoid preparation was measured at this stage, and dilution with buffer made to a standard difference of 0·00400 between the refractive index n of the protein solution, and n_0 that of the buffer. Mobility measurements were made in solutions of half this strength. The buffer usually employed was a phosphate buffer (pH 8·0, ionic strength $\mu = 0{\cdot}1$). Buffers used in mobility determinations were phosphate buffers, $\mu = 0{\cdot}1$, except for pH values below 6, where acetate buffers were necessary, and at values above 8, where borate buffers were used.

Electrophoretic technique. Runs were carried out in the Tiselius (1937) apparatus at +0·5°. The potential gradient in the **U** tube was held constant, usually at some value in the range 1·5–3·5 V./cm. The diagonal *schlieren* method (Philpot, 1938) was employed, using as light source a mercury vapour lamp from which monochromatic light ($\lambda = 546$ mμ.) was isolated by means of a filter. The curves were photographed on Kodak Half-Tone (Wratten) Panchromatic Plates (*P* 25).

Measurements of pH were made with a double hydrogen electrode, using 0·1 N-HCl (pH 1·075) as the standard reference solution. Conductivity measurements on the buffers, necessary for mobility calculations, were made in a Washburn-type conductivity cell, and the distances moved by the components were measured from enlargements (of known magnification) obtained by tracing on graph paper the outlines of the patterns projected from the plates in a photographic enlarger.

A few runs, for the purpose of investigating different fractions, were carried out in the large type of **U** tube as employed by Blix, Tiselius & Svensson (1941).

RESULTS

Electrophoretic patterns of various diphtheria toxoid preparations

Typical curves are shown in Fig. 1 *A–F*. These indicate generally two major components, present in rather different relative amounts in the different preparations. A quantitative estimate was not attempted since the separation of the two peaks could not be satisfactorily achieved for this purpose. Examination of the contours of the curves also reveals that, in some cases, these components are themselves undoubtedly composite (e.g. see Fig. 1 *A*, *B*). Another feature, most prominent in Fig. 1 *E*, *F*, is a sharp spike projecting from the stationary ϵ boundary (for the origin of the ϵ boundary see Longsworth & MacInnes, 1939). At one period there was a suggestion that antigenicity might be associated with the material responsible for this peak, but an extension of the observations to a larger number of toxoid preparations (about 30) showed that this could not be correct. The correlation of electrophoretic patterns with the antigenic response in horses was further complicated by the large variation in the responses of different horses to the same batch of toxoid.

The curves shown in Fig. 1 were all obtained from toxoids produced on papain digest media.

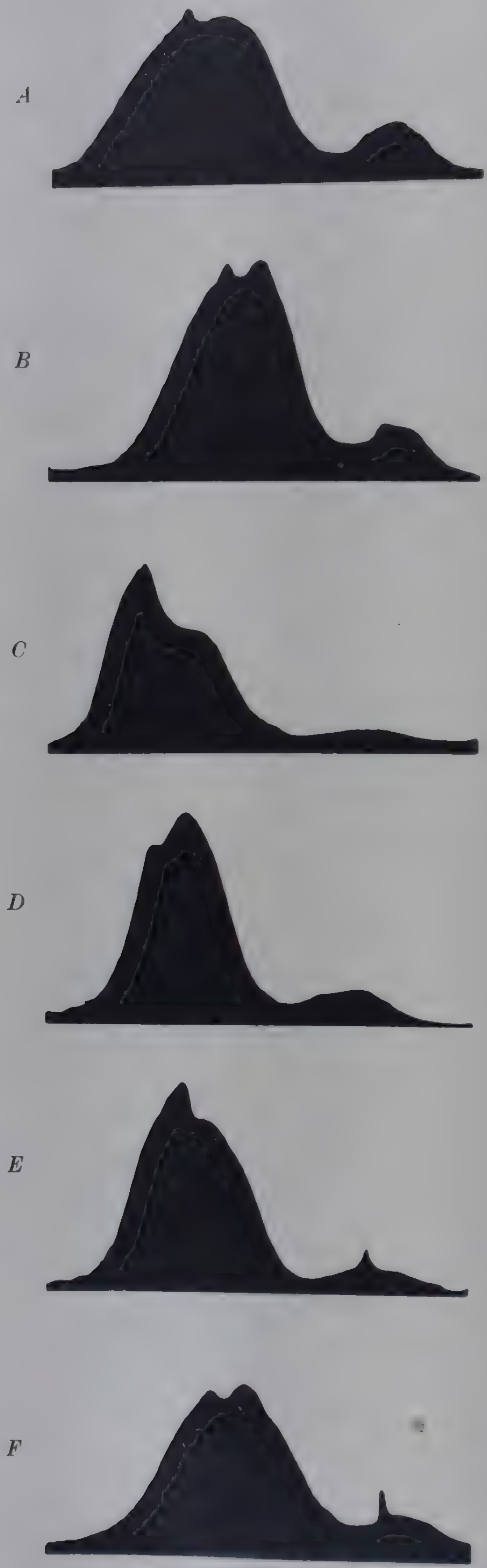

Fig. 1. Electrophoretic patterns of typical diphtheria toxoid preparations (see text) in phosphate buffer pH 8, $\mu = 0{\cdot}1$, 2·6 V./cm. Exposures approximately 2 hr. after starting current. Only cathode limb curves are recorded. Migration is from right to left. All these toxoids were produced from a papain digest of beef medium.

Fig. 2 shows the patterns *A* and *B*, obtained from toxoids produced on an acid hydrolysate of casein and a tryptic digest of beef medium respectively. The latter shows the presence of a new component. Methods of purification similar to those described by Pope & Linggood (1939) and Linggood (1939) were attempted. Treatment of the tryptic digest toxoid with 5 % Farnell's *L.S.* carbon (Farnell Carbons Ltd., 1A Conduit Road, S.E. 18) removed most of this new component (see Fig. 2*C*). Treatment with 7 % Farnell's *L.S.* carbon followed by precipitation from the filtrate by addition of 15 % (v/v) of 5 % (w/v) cadmium chloride solution, and subsequent extraction from the precipitate by 2 % (w/v)

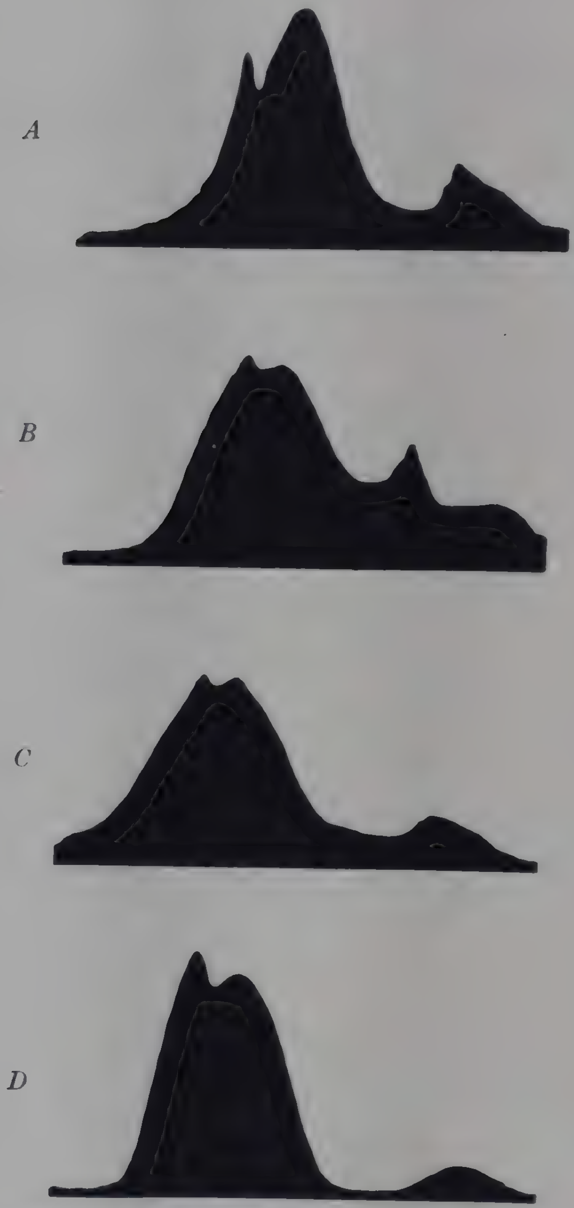

Fig. 2. Electrophoretic patterns of diphtheria toxoid preparations in phosphate buffer pH 8, $\mu = 0{\cdot}1$, 2·8 V./cm. Exposures approximately 2 hr. after starting current. Only cathode limb curves are recorded. Migration is from right to left. Details of these preparations are mentioned in the text.

$Na_2HPO_4.12H_2O$ solution gave the product shown in Fig. 2*D*. The components here appeared to be purer, judging by the regular contours of the peaks.

Treatment of ordinary (papain digest) toxoids with 5 % Farnell's *L.S.* or Sutcliffe, Speakman's *P.Z.* charcoal (Sutcliffe, Speakman and Co. Ltd., Leigh, Lancs.) showed that the electrophoretic pattern was scarcely affected.

Ammonium sulphate fractionation of concentrated toxoid produced no significant alteration in the relative amounts of the components present, either in the filtrate or in the precipitate.

A method of purification developed by Dr C. G. Pope, to be described elsewhere, produced a significant alteration in the electrophoretic pattern. A sample of toxoid purified by this method and assaying at 1700 *Lf* doses in each mg. of protein nitrogen gave, at pH 5·5, the pattern shown in Fig. 3. It consists of approximately 80 % of slow-moving material together with smaller amounts of faster-moving impurities. This pattern should be compared with that given by an untreated toxoid at the same pH (see Fig. 5).

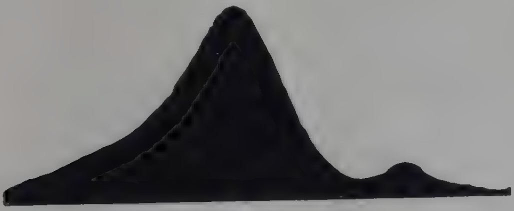

Fig. 3. Electrophoretic pattern of a purified diphtheria toxoid preparation in acetate buffer pH 5·5, 3·4 V./cm. Exposure 199 min. after starting current. The cathode limb is shown. Migration is from right to left.

Investigation of electrophoretically separated fractions

Runs were carried out in both the ordinary and the large **U** tube, to obtain, if possible, fractions representing one or other of the components present in toxoids. Isolation of fractions was carried out by hydrostatic displacement of the boundaries to the requisite position followed by sectioning off the **U** tube compartments which could be subsequently emptied independently. First, as regards the material responsible for the small, sharp, nearly stationary peak in some toxoids (e.g. see Fig. 1*E*) solutions containing only this component were isolated, analyzed for total nitrogen, and tested for activity by flocculation. Samples from other sections of the **U** tube were also tested as a check that toxoid activity had not been greatly diminished during the course of the experiments. The strongest solution of the minor component thus obtained gave total N 40·8 μg./ml. (allowing for a blank on the buffer), but no *Lf* units could be detected. Thus, this component could not possess activity of more than about 25 *Lf* units/mg. N and would therefore be able to make only a very small contribution, if any, to the *Lf* units present.

In a similar manner solutions containing the fastest component were isolated and examined. Again, when care was taken to ensure that this component was obtained free from others, no toxoid activity was detectable in it by flocculation testing, although solutions containing nitrogen equivalent to 0·5–1·0 % protein were isolated. It would thus appear that the toxoid activity is associated with the slower of the main components.

To test this conclusion a mixture of a toxoid with an equivalent amount of antitoxin (known, from previous electrophoretic work, to contain only β and γ globulins) was flocculated. The floccules were spun off and the supernatant liquid, concentrated and washed with buffer on an ultrafilter, then submitted to electrophoresis. The pattern obtained showed that most of the residual protein consisted of the inactive portion of β, γ globulins; but there was also some material which corresponded with the faster major component of the toxoid. This result was in accord with the experiments just described.

Mobility measurements. Mobility measurements were made over the range of pH values (5·0–9·0) throughout which the toxoids are fairly stable. Calculations were made only for the major components, and only from descending boundary rates (see Longsworth & MacInnes, 1940). Values of the mobilities for both components varied slightly from one preparation to another. Typical results are shown in Fig. 4. Each point is the mean of two determinations on the same solution. The curves can be extrapolated to indicate that the isoelectric points are approximately in the region of pH 3·5–4·0, as is known from other data.

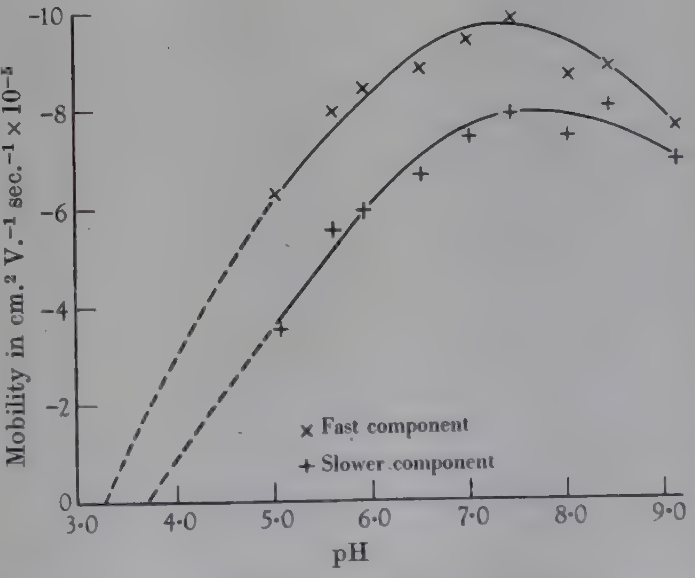

Fig. 4. Electrophoretic mobilities of diphtheria toxoid components in the pH range 5·0–9·0. Values are uncorrected for buffer viscosities.

Below a pH of about 6 there was a greater tendency for the composite nature of the peaks to be emphasized; thus at pH 5·5 the slower major peak can be seen to possess a very distinct hump, indicative of a boundary due to material which is moving rather more slowly (see Fig. 5).

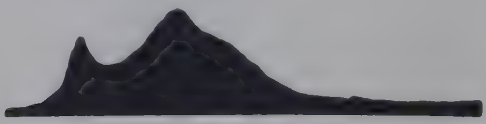

Fig. 5. Electrophoretic pattern of a diphtheria toxoid preparation in acetate buffer pH 5·5, 1·7 V./cm. Exposure 335 min. after starting current. The cathode limb is shown. Migration is from right to left.

DISCUSSION

Some discussion of the sharp peak appearing in the electrophoretic pattern of certain toxoid preparations appears to be called for. It is somewhat reminiscent of effects produced by convection and of the β-anomaly (Longsworth, Shedlovsky & MacInnes, 1939). Reasons for assuming that it is not due to convection are (*a*) the detection of non-dialyzable nitrogen in this boundary, (*b*) the reproducible nature of the phenomenon with a given toxoid. Thus it was found that when a particular toxoid was divided into separate samples and submitted to electrophoresis, the curves obtained either all failed to exhibit this peak or all showed it; and, when present, it was in the same position and moved at the same slow rate. Repeated washing with buffer, on the ultrafilter, did not cause this peak to vanish from the pattern of any toxoid which originally showed it.

An unusual feature is the shape of the mobility curves, the speed of migration attaining a maximum value in each case at about pH 7·0–8·0. Although the mobilities are uncorrected for buffer viscosities, the corrections would not cause differences large enough to remove the effect of these maxima. A similar effect has recently been observed by Watson, Cromartie, Bloom, Heckly, McGhee & Weissman (1947) in the case of glutamyl polypeptide, etc. However, in their work the effect appeared only in the results obtained from the anode (ascending) limb, which is known to give anomalous results (Longsworth & MacInnes, 1940). It appears highly probable that the causes of the maxima are similar in the two cases, due to interaction between the molecules of the substance under examination, or to interaction with the buffer.

SUMMARY

1. Electrophoretic patterns of diphtheria toxoid preparations have been obtained, and typical curves are shown.

2. Most preparations show two major peaks with, in some cases, a slow-moving minor peak. The components represented by the major peaks, judging by their contours, are not quite homogeneous.

3. One preparation, purified chemically, appeared to be about 80% pure.

4. Toxoid activity has been assigned entirely, or almost entirely, to the slower major component.

5. The mobilities of the two major components have been measured over a pH range of 5·0–9·0.

6. Attempts to correlate the electrophoretic pattern with antigenicity have so far failed.

The author wishes to express his thanks to Dr F. V. Linggood and to Dr C. G. Pope of these laboratories, the former for suggesting this investigation, and the latter for discussions during the course of the work. He is also indebted to Mr E. Fenton for technical assistance in the preparation of the materials for electrophoresis.

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New Zealand Fish Oils

4. OBSERVATIONS ON THE OIL CONTENT OF FRESH WATER EELS

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(*Received* 25 *August* 1947)

Two species of fresh water eel *Anguilla australis schmidtii* Phillips and *A. dieffenbachii* Gray are known to occur in New Zealand, where they are conveniently termed respectively, short-finned and long-finned eels. Although the former species has been comparatively neglected apart from the determination of the vitamin A and vitamin D content of the body oil (Denz & Shorland, 1934; Cunningham, 1935; Weeber, 1945), the latter species has received the attention of several investigators in respect of the fatty-acid composition of the body oil (Shorland & McIntosh, 1936) and distribution of vitamin A and oil in the tissues (Edisbury, Lovern & Morton, 1937). The contributions made by the latter investigators as well as by Lovern (1938) on the European eel, *A. anguilla* L., suggest that the oil content increases with the length of the fish. McCance (1944), however, denied that the oil content could be foretold from the length, and his data showed that the oil content of the migrant eel, irrespective of length, was relatively constant. In regard to the immature eels it is our belief that no generalization was justified as he used only two samples and, although our results agree with those of McCance in respect of migrant eels, the results for immature eels support Lovern's (1938) contention that the oil content increases with the length of the eel.

EXPERIMENTAL

The material used in this work was collected from Lake Ellesmere migrant populations in 1942 and from immature eels from that lake in 1943 and 1944 as well as from Foxton Lakes, 1943, Manawatu River, 1943, and Hutt River, 1943. To eliminate the possibility of seasonal effects the samples were taken during the months of March and April. The eels were divided into length groups, each group from each locality containing usually 5–10 eels commencing with the length group 22 in. and under, and proceeding by 3 and 4 in. intervals respectively for short-finned and long-finned eels, up to the largest sizes obtained. This yielded four groups of short-finned eels and four to six groups of immature long-finned eels. The migrant long-finned eels were in general longer than the immature specimens, and were divided into four groups, covering a range of 39–55 in. General consideration of the length-weight relationships will be published elsewhere, but it may be stated here that the same species of eel showed similar relationships in the various localities studied.

The eels from each group were subdivided into head, trunk, tail, liver, viscera (excluding liver) and ovary, when the latter was present. The head was severed at the first vertebra, while the trunk and tail portions were separated by a transverse cut at the vent.

RESULTS

The proportions of the various tissues in the eels examined are shown in Table 1.

In both short-finned and long-finned species, the immature eels from Foxton Lakes are shown to have heads which are significantly larger than those from other localities studied. Other locality differences of a lesser degree may also be noted in the proportions of other tissues of the immature eels. Comparing migrant with immature eels it will be seen that the changes in tissue proportions are confined in the case

Table 1. *Proportions of the dissected tissues of eels expressed as a percentage of the total weight*

Source	Head	Trunk	Tail	Ovary	Viscera	Liver
			Short-finned eels			
†Lake Ellesmere, 1943	4·79 ± 0·61*	43·19 ± 1·52	45·93 ± 0·32	—	5·16 ± 0·78	0·93 ± 0·05
†Lake Ellesmere, 1944	5·04 ± 0·50	43·49 ± 0·62	45·67 ± 0·77	—	5·08 ± 0·80	0·72 ± 0·04
†Foxton Lakes, 1943	9·84 ± 1·38	41·91 ± 1·19	43·59 ± 1·36	—	4·11 ± 0·50	0·55 ± 0·02
†Hutt River, 1943	4·07 ± 0·27	41·91 ± 0·76	49·38 ± 1·28	—	3·55 ± 0·37	1·09 ± 0·06
‡Lake Ellesmere, 1942	4·8 ± 0·43	43·30 ± 1·17	45·60 ± 0·90	3·5	*c.* 1·83	0·97 ± 0·07
			Long-finned eels			
†Lake Ellesmere, 1943	9·08 ± 1·01	46·05 ± 0·61	37·02 ± 1·18	0·64	6·17 ± 0·34	1·04 ± 0·01
†Lake Ellesmere, 1944	7·42 ± 1·16	44·79 ± 0·89	41·79 ± 2·11	—	5·24 ± 0·46	0·76 ± 0·06
†Foxton Lakes, 1943	12·96 ± 0·24	42·16 ± 1·37	39·12 ± 1·38	—	4·78 ± 0·39	0·98 ± 0·18
†Hutt River, 1943	7·42 ± 0·87	42·72 ± 0·88	43·17 ± 1·60	—	5·51 ± 0·43	1·18 ± 0·06
†Manawatu River system, 1943	10·18 ± 0·38	39·12 ± 1·72	44·42 ± 2·23	—	5·26 ± 0·46	1·02 ± 0·05
‡Lake Ellesmere, 1942	5·5 ± 0·4	43·2 ± 1·28	36·9 ± 1·26	10·7 ± 0·8	2·0 ± 0·31	1·7 ± 0·05

* Standard error. † Immature eels. ‡ Migrant eels.

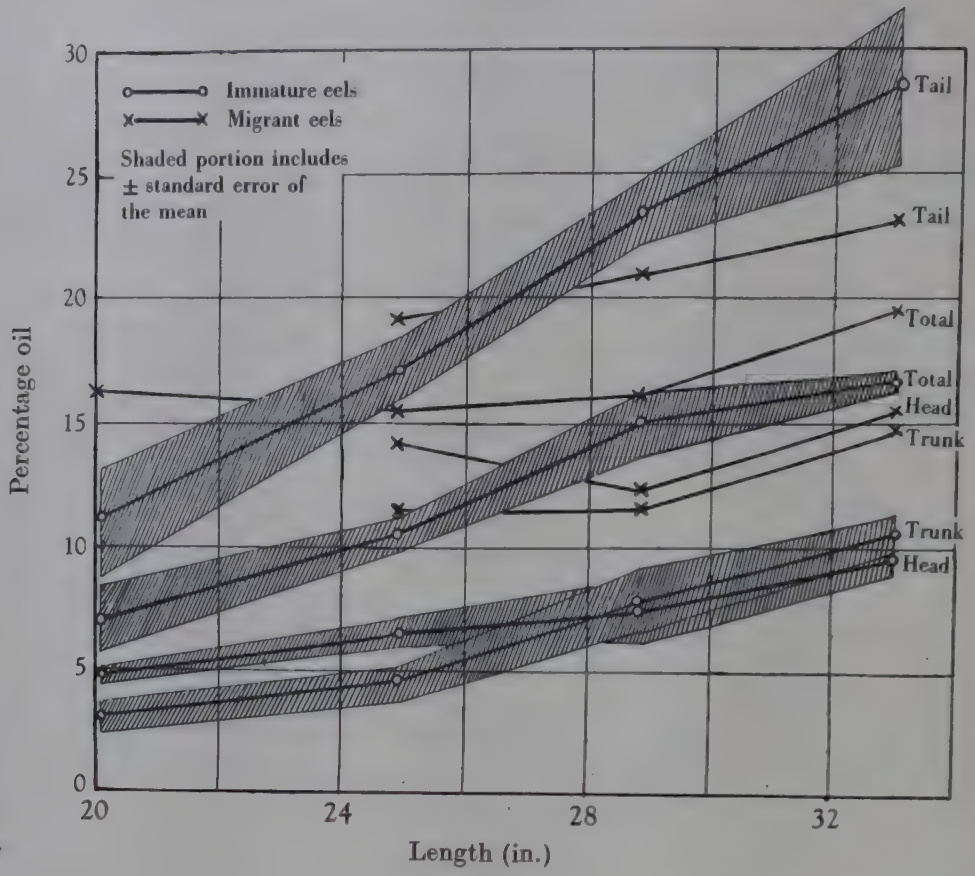

Fig. 1. Oil content of tissues of *A. australis.*

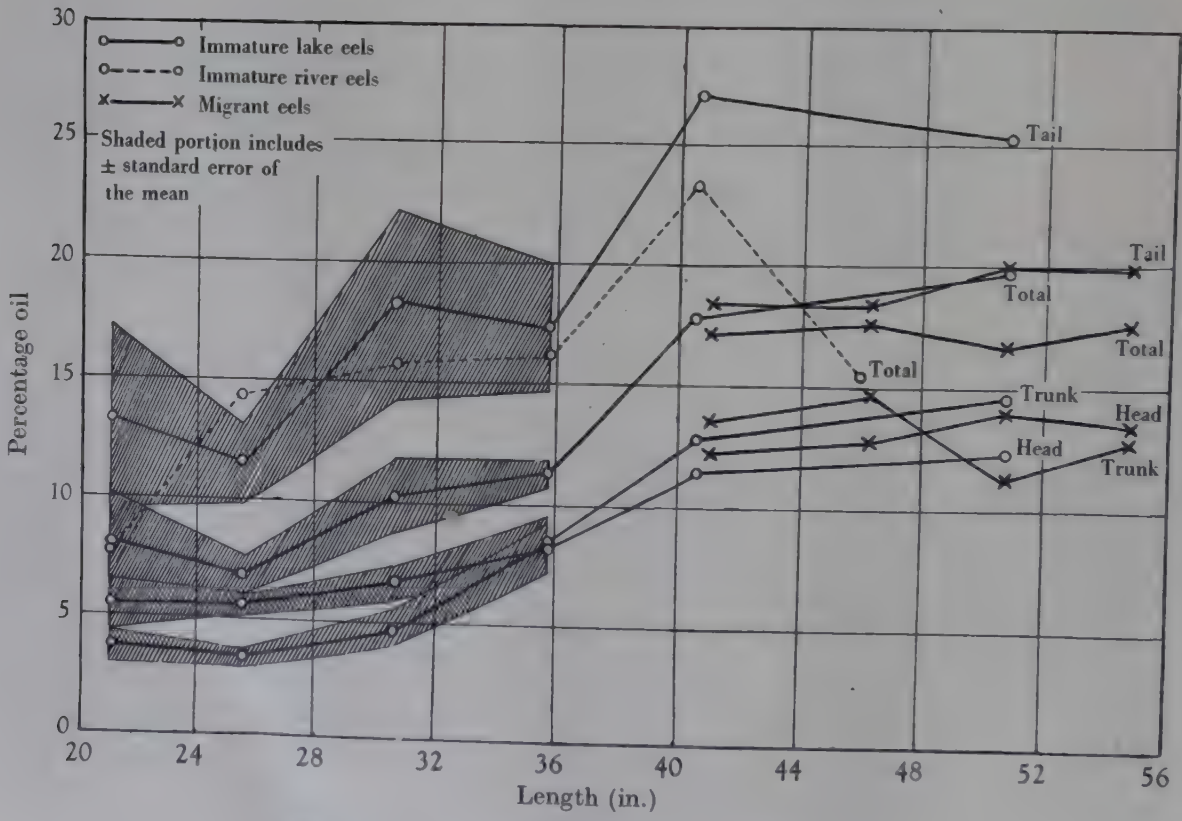

Fig. 2. Oil content of tissues of *A. dieffenbachii.*

of the short-finned eels to the development of 3·5 % of ovary with a corresponding reduction in the amount of viscera. In the long-finned species the transition from the immature eel to the migrant is accompanied by a relatively greater production of ovary (10·7 %) and a significant increase in the proportion of liver, with a decrease in the amount of head.

The oil content of the various tissues of the eels is given in Figs. 1 and 2. As no significant differences were observed between the oil content of similar groups of short-finned eels from different localities the separate results for the percentage total oil and percentage oil in the dissected tissues have been respectively combined. For similar reasons results for long-finned eels from Foxton Lakes and Lake Ellesmere have also been combined, while the average total oil content of the Manawatu and Hutt River specimens, which were significantly oilier, are shown separately. Since, however, the results for the oil content of the tissues of the long-finned eels from the Manawatu and Hutt Rivers showed similar variations to those exhibited by the Foxton Lakes and Lake Ellesmere specimens, they have been omitted from Fig. 2.

It will be observed from Figs. 1 and 2 that the oil content of immature eels tended to increase with the length of the fish in agreement with Lovern's (1938) observations on *A. anguilla* L. This tendency, however, appeared to be confined to the immature eel, the migrant showing little, if any, increase in oil content with length.

The general picture of the relationship between the oil content and length of both species is shown to be somewhat similar, but the long-finned eels may be distinguished from the short-finned by their greater variability in the oil content of the tail as well as by their less uniform relationship between total oil content and length. Attention is drawn to the interesting relationship between the oil content of the head and trunk which was found to be similar in both species. In the smaller immature specimens the oil content of the head exceeds that of the trunk, but with the larger immature specimens the reverse is true. In the migrant eel the situation in regard to the relative oil content of the head and trunk is shown to revert back to that of the smaller immature eel.

Certain differences may be noted in respect of the oil content of the two species. The short-finned migrant is shown to contain (cf. Fig. 1) a higher percentage of total oil than the immature eel of the same length, while in the case of the long-finned species (cf. Fig. 2) the reverse is true. In the short-finned species the migrant, as compared with the immature eel of the same length, tended to have a lower percentage of oil in the tail but a higher percentage in the head and trunk. In this connexion the long-finned species resembled the short-finned as regards the tail oil, but differed in respect of the trunk and head oil percentages which showed relatively small variation between migrant and immature specimens of the same length (Table 2).

Table 2. $\dfrac{\textit{Percentage of oil in tail}}{\textit{Percentage of oil in head}}\left(\dfrac{T}{H}\right)$ *and* $\dfrac{\textit{Percentage of oil in tail}}{\textit{Percentage of oil in trunk}}\left(\dfrac{T}{F}\right)$ *in immature and migrant eels*

	Immature eels		Migrants	
	T/F	T/H	T/F	T/H
Short-finned	3·42 ± 1·06*	2·80 ± 0·84	1·69 ± 0·13	1·51 ± 0·19
Long-finned	3·29 ± 1·13	2·54 ± 0·85	1·47 ± 0·20	1·47 ± 0·06

* Standard deviation.

In considering the ratios T/F and T/H in Table 2, immature long-finned specimens of over 38 in. have been excluded, as such specimens may contain appreciable proportions of ovary. The ratios T/F and T/H show a much greater uniformity in the migrant eels as compared with immature eels, as well as a more uniform distribution of oil in the former as compared with the latter. The results shown in Figs. 1 and 2 are consistent with the hypothesis that greater uniformity in the distribution of oil in the migrant, as compared with the immature eel, is achieved by means of a considerable movement of oil from the tail to the trunk, followed by a further movement of oil from the trunk to the head. In the long-finned species, however, the situation is complicated by the development of the ovary as an important oil reserve (cf. Fig. 1), and it is suggested that, comparing long-finned species with short-finned, the relatively low proportion of oil in the trunk and head sections of the migrant as compared with the immature long-finned eel is due to a further considerable diversion of the trunk oil into the ovary.

In Figs. 3 and 4 the oil contents of the tissues are expressed as a percentage of the total oil reserves and compared with the proportions of these tissues in the fish, which, in the case of the short-finned eels were not significantly different as between migrant and immature eels, apart from the replacement in the former of the viscera by ovary. As no significant differences in oil distributions were found between groups of long-finned eels below 38 in. used in this work, the results for these groups have been combined as shown in Fig. 4.

In the immature short-finned eel, the tail, comprising 46 % of the total weight, contained 75 % of the total oil in the fish. With the onset of maturity the amount of oil in the tail was reduced to 57 % of the total oil reserves. In the immature long-finned eel the tail portion amounted to 41 % of the total weight and contained 68 % of the total oil reserves. At maturity the tail was slightly reduced in amount to 37 % with a spectacular reduction in oil content to 41 % of the total oil reserves, coinciding with the appearance of the ovary as an important oil depot, which accounted for 20 % of the total oil reserves in the fish as compared with only 7 % in the mature short-finned eel. Although the proportion of trunk (42–43 %) in both the short-finned and long-finned eels did not change as the fish matured, the oil reserves of this section of the fish, expressed as a percentage of the total oil reserves, increased respectively from 19 to 32 % and from 23 to 33 %.

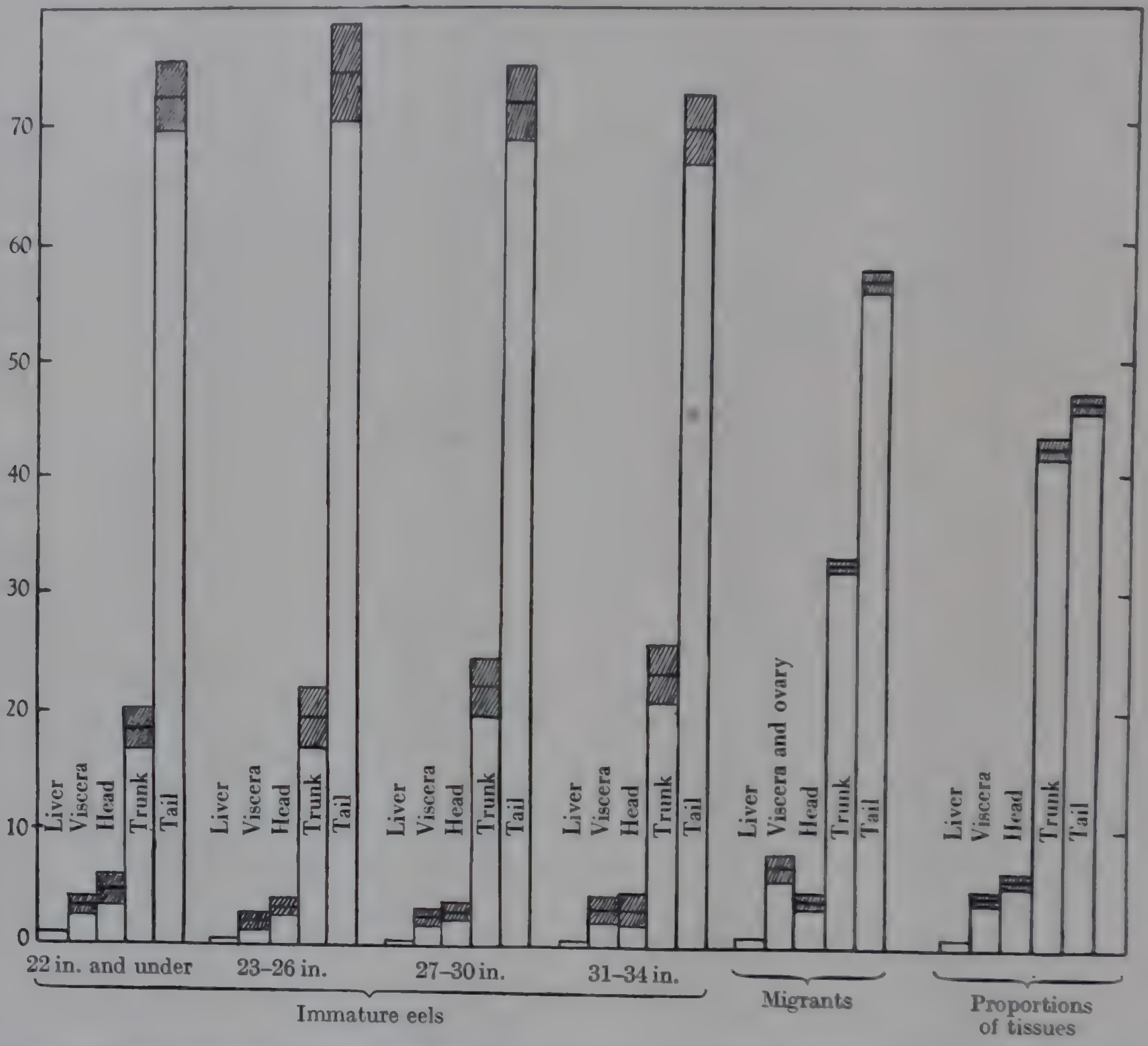

Fig. 3. Distribution of oil in the tissues of *A. australis* expressed as a percentage of the total oil. (Shaded portions include ± standard error.)

SUMMARY

1. It was found that commencing with eels of *c.* 50 cm. length and proceeding to fully adult migrant eels the oil content of the immature eels tended to increase continuously with length from approximately 7 to 17 % in *A. australis* and from 7 to 23 % in *A. dieffenbachii.*

2. The largest size of immature eel was found to approximate in oil content to that of the migrant eel, the oil content of which was not found to vary appreciably with the length of the fish.

3. Considering the tissues separately, however, it was found that whereas the distribution of oil was fairly uniform in the migrant eel as between the head, trunk and tail, this was not so in the case of the immature eel which contained approximately 70 % of the total oil reserves in the tail comprising but 42–43 % of the total weight of the fish.

4. The observed results are consistent with the theory now put forward that just prior to migration a more uniform distribution of oil is attained by a partial transference of oil from the tail into the trunk and from thence into the head and ovary.

We wish to thank Mr A. E. Hefford, M.Sc., formerly Chief Inspector of Fisheries and Mr D. F. Hobbs, Senior Fresh Water Biologist to the Marine Department for encouragement and for generous assistance in the collection of samples.

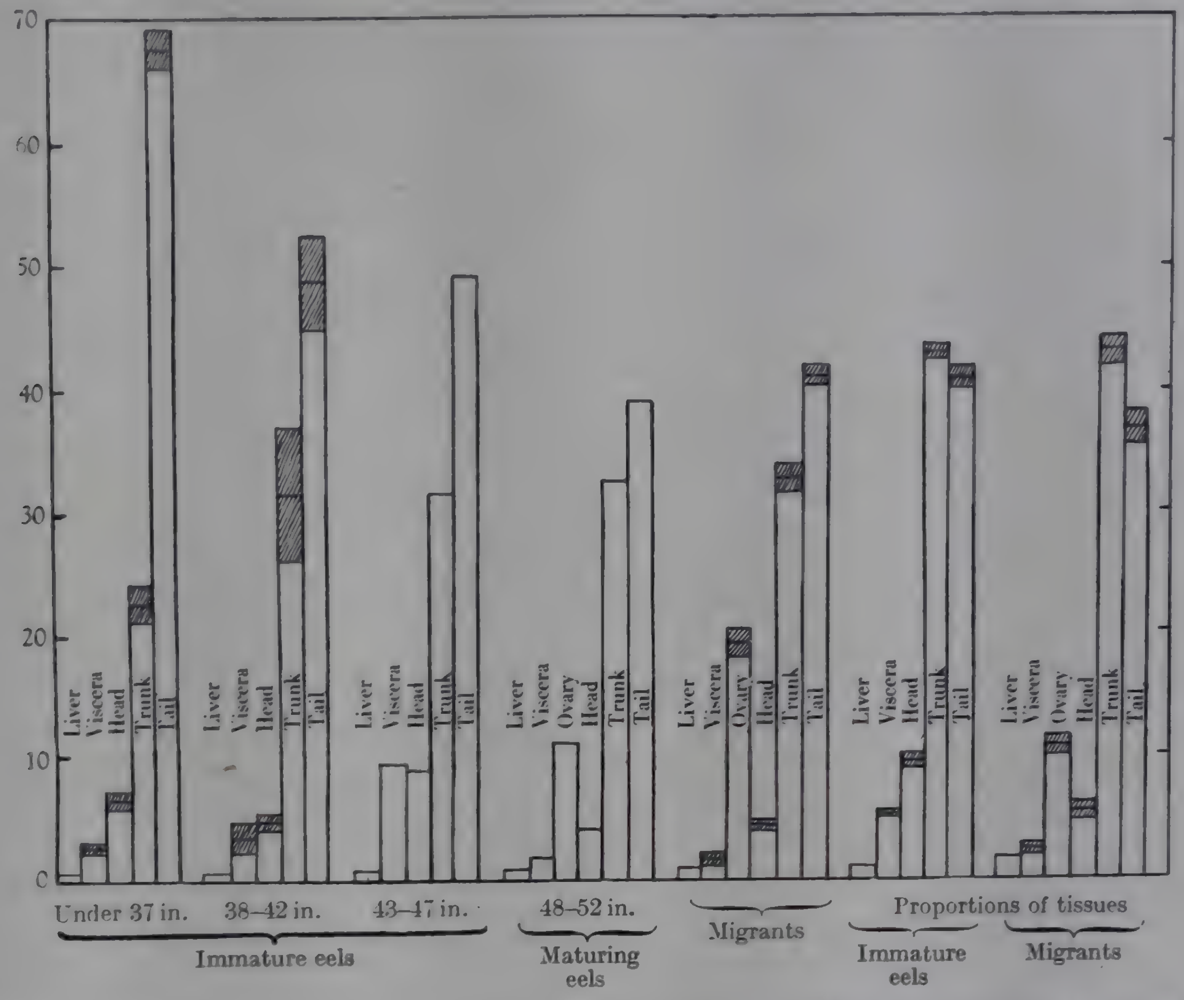

Fig. 4. Distribution of oil in the tissues of *A. dieffenbachii* expressed as a percentage of the total oil. (Shaded portions include ± standard error of the mean.)

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The Fate of Certain Organic Acids and Amides in the Rabbit

4. THE AMINOBENZOIC ACIDS AND THEIR AMIDES

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Investigations of the metabolism of the aminobenzoic acids in mammals have suggested that the principal methods by which these compounds are conjugated *in vivo* are acetylation of the amino group and conjugation of the carboxyl group with glucuronic acid. For example, Muenzen, Cerecedo & Sherwin (1926) found that *m*- and *p*-aminobenzoic acids, but not anthranilic acid, were acetylated by man and the rabbit, although none of the acids was conjugated in this way by the dog. Acetylation of *p*-aminobenzoic acid has also been observed in the rabbit by Ellinger & Hensel (1914), Hensel (1914), and Harrow, Mazur & Sherwin (1933) and in man and the guinea pig by Zehender (1943). Mitsuba & Ichihara (1927) reported that anthranilic acid was the only aminobenzoic acid to cause an increased excretion of reducing substances in the dog or rabbit, although Quick (1932) showed that in the dog all three isomers were conjugated to a considerable extent with glucuronic acid.

Other methods of conjugation are, however, recorded in the literature. Mitsuba & Ichihara (1927) found that anthranilic acid, but not the *m*- or *p*-isomers, gave rise to an increased excretion of ethereal sulphate by the dog and rabbit. Without satisfactory evidence they attributed this increase to the formation of salicylic acid. Salkowski (1882) also observed no increase in ethereal sulphate excretion when *m*-aminobenzoic acid was given to dogs or rabbits and claimed to have isolated some *m*-aminohippuric acid from the urine. He also stated that some *m*-ureidobenzoic acid (*N*-phenylurea-3-carboxylic acid) was formed. Hildebrandt (1903) claimed that this type of conjugation occurred with all three isomers in the rabbit and mouse, and Mitsuba & Ichihara (1927) confirmed this for anthranilic acid in the dog and rabbit. Muenzen *et al.* (1926) however, denied the formation of ureido- and aminohippuric acids in man, dog or rabbit although they gave little detail to support their statement. Formation of other ureido compounds *in vivo* has been suggested by early workers, e.g. from taurine in man and dog (Salkowski, 1872, 1873) and from sulphanilic acid in the dog (Ville, 1892); but Schmidt & Allen (1920) were unable to detect the excretion of ureidotaurine as a metabolic end product of taurine in man, and we have not obtained *p*-ureidobenzenesulphonic acid as a metabolite of sulphanilic acid in the rabbit (unpublished). In the present work, although we did not deliberately search for ureido acids, we have not made any observation which suggests their formation. Several workers have recorded that the aminobenzoic acids are partly excreted unchanged, e.g. Salkowski (1882) (*m*-isomer), Hildebrandt (1903) (all isomers), Muenzen *et al.* (1926) (*o*-isomer only in man and rabbit, all isomers in dog).

The metabolism of the aminobenzamides has received little attention. The hypothesis (Woods, 1940; Fildes, 1940) that the bacteriostatic effect of sulphanilamide is due to its structural similarity to *p*-aminobenzoic acid which enables it to compete with that compound in certain essential metabolic processes in bacteria led to the expected observation (Hirsch, 1942) that *p*-aminobenzamide had similar bacteriostatic activity to that of sulphanilamide. In the work reported here we have compared the fate of the aminobenzamides in the rabbit with that of their respective acids. The extent of conjugation with acetic, glucuronic and sulphuric acids and the excretion of ether-soluble acid show that, unlike the carbamyl group in benzamide (Bray, Neale & Thorpe, 1946*a*), the same group in aminobenzamides is comparatively stable to hydrolysis. One conspicuous difference between the acids and amides is that the latter are oxidized to aminohydroxybenzamides which are at least to some extent conjugated with sulphuric acid, whereas the acids are not oxidized, the principal conjugates excreted being ester-type glucuronides. Some of these metabolites (or derivatives of them) have been isolated and identified. The aminohydroxybenzoic acids isolated* support the view put forward in an earlier paper (Bray, Ryman & Thorpe, 1947) that when oxidation of a disubstituted aromatic compound is effected by the introduction of a hydroxyl group into the ring, the resulting phenol has a 1:3:4-configuration, although the isolation of 3-hydroxyanthranilic acid as an oxidation product of *o*-aminobenzamide makes it clear that the hypothesis must be extended to include such compounds.

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* All oxidation products isolated in this work were acids. It is not known whether the actual compounds excreted are acids or amides. It is very probable that in the case of the *o*-isomer the amide is excreted, since the percentage oxidation is greater than the estimated degree of hydrolysis, but analytical data for the other isomers do not allow any similar deductions to be made. In this paper the term 'oxidation product' may apply to either acid or amide.

METHODS

Preparation of aminobenzamide. The amides used were prepared by reduction of the corresponding nitrobenzamides using $FeSO_4$ and NH_3 (Jacobs & Heidelberger, 1917). The nitroamides were prepared by the action of ammonia upon the acid chlorides.

Diet and feeding. The rabbits used were does of 2–3 kg. maintained on a constant diet of rabbit pellets (Bruce & Parkes, 1946), except in the quantitative study of *p*-aminobenzoic acid and amide when bran and oats were used (for details of administration of the diets see Bray *et al.* 1947). We have no reason to believe that the metabolism of these compounds differs in any essential way on the two diets, except for variations in the 'base line' values of some normal metabolites as described below.

Dosage. Owing to their toxicity, anthranilic acid and its amide were not given in doses exceeding 0·5 g./kg. The other acids and amides were not toxic at dose levels up to 1 g./kg. The compounds were administered by stomach tube, the acids being dissolved in $NaHCO_3$ solution and the amides suspended in water.

Estimation of ether-soluble acid. The method was essentially that previously described (Bray *et al.* 1946*a*). We found that all three acids could be quantitatively extracted from water or urine at pH 4. This pH was attained in the urines by adding to 10 ml. urine 10 ml. of a solution containing no ether-soluble material and made up by mixing 40 ml. M-Na_2HPO_4 with 52 ml. M-$NaHSO_4$.

Estimation of diazotizable material. This was carried out according to the method of Bratton & Marshall (1939) as referred to previously (Bray *et al.* 1946*b*). Eckert (1943) states that in blood filtrates quantitative estimation of *p*-aminobenzoic acid requires 15–20 min. for diazotization and 30–60 min. for coupling. With our solutions we did not find any modification of the Bratton-Marshall procedure necessary. With the *o*-acid and amide, however, it was necessary to leave the coupled solution 22 hr. in order to attain maximum colour development. Kohl & Flynn (1941) state that a diazotization procedure using 3 hr. for colour development gives low values for anthranilic acid after hydrolysis, owing to the formation of an undiazotizable anhydride, anthranil, when solutions are heated at an acid reaction. We found that if 22 hr. were allowed for colour development, solutions of anthranilic or acetylanthranilic acids, which had been subjected to the usual hydrolysis procedure, gave values within 3% of the theoretical.

Anthranilic acid gives rise to a much more intense colour than an equivalent amount of the corresponding amide. To match 5·8 mg. of the former 10 mg. of the latter are needed. The conditions of hydrolysis used (1 hr. with 0·2 N-HCl in a boiling water bath) are not sufficiently drastic to cause significant hydrolysis of the carbamyl group. It has already been reported (Bray, James, Ryman & Thorpe, 1948) that only 8% hydrolysis of the amide occurs on heating with N-HCl in a boiling water bath for 1 hr. Solutions of *o*-aminobenzamide and its *N*-acetyl derivative subjected to the ordinary hydrolysis procedure gave quantitatively accurate results when compared with a standard untreated *o*-aminobenzamide solution. An amide standard was, therefore, always used in determinations on urines obtained after administration of *o*-aminobenzamide. It will be seen later that some hydrolysis of the carbamyl group occurs *in vivo*. The values obtained for the percentage of *o*-aminobenzamide excreted with a free amino group (Table 2) will therefore appear higher than they actually are. The oxidation products isolated after acid hydrolysis following the administration of *o*-aminobenzamide, 3- and 5-hydroxyanthranilic acids, give no appreciable colour upon diazotization and coupling under our conditions of estimation. This probably applies also to their amides, which are likely to be the compounds actually excreted. Stronger solutions of these acids (100–200 mg./100 ml.) give a violet colour on diazotization and coupling (see p. 440).

The oxidation products isolated from *m*-aminobenzamide urine after acid hydrolysis likewise give no diazo reaction. The difference in colour intensity of the diazotized and coupled acid and amide is much less than with the *o*-isomer (9·4 mg. acid ≡ 10 mg. amide), but is sufficient to require the use of an amide standard.

The colours developed from *p*-aminobenzoic acid and its amide are of equal intensity, but an amide standard was actually used in experiments in which *p*-aminobenzamide was given. This amide is only oxidized to a very small extent and the oxidation product (isolated as acid) gives no diazo reaction.

Estimation of reducing substances. This was carried out as previously (Bray *et al.* 1946*b*). The method does not give reliable results for ether-type glucuronide excretion if the amount of ester-type glucuronide excreted is high, as in the case of the aminobenzoic acids. Further, oxidation products may interfere if they are themselves reducing. Since the aminohydroxybenzoic acids isolated after the administration of *o*- and *m*-aminobenzamides are strongly reducing, values for these amides are probably unreliable. Values obtained with the *p*-amide can be accepted since it is only oxidized to a very slight extent.

Results of estimations of ester-type glucuronides formed from the aminobenzoic acids are probably somewhat low. Pure *p*-aminobenzoylglucuronide (see p. 439) gave values about 9% lower than the theoretical.

Estimation of ethereal sulphate. This was carried out as previously, by the method of Folin (1905–6).

EXPERIMENTAL AND RESULTS

Quantitative studies

Ether-soluble acids. The ether-soluble acid of the normal urine of rabbits on the bran, oats and cabbage diet ranged from 200 to 450 mg./day (calculated as hippuric acid) and on the pellet diet from 400 to 700 mg. Average values were 325 and 574 mg. respectively. The results obtained after administration of aminobenzoic acids and amides (calculated as aminobenzoic acid) are summarized in Table 1.

It should be noted that the ether-soluble acid values obtained represent only acid unconjugated with glucuronic acid since the glucuronides are not extracted by ether. Thus, the difference between the ether-soluble acid values for

Table 1. *Excretion of ether-soluble material (calculated as aminobenzoic acid) by rabbits after administration of aminobenzoic acids and amides*

	Percentage of dose excreted as ether-soluble acid								
	o-Compound			*m*-Compound			*p*-Compound		
Dose (g./kg.)	Rabbit no.	Acid	Amide	Rabbit no.	Acid	Amide	Rabbit no.	Acid	Amide
0·25	131	60	36	—	—	—	133	45	29
	135	61	27	—	—	—	125	29	10
	137	79	38	—	—	—	137	20	Nil
	144	69	31	—	—	—	139	26	10
0·5	131	29	4	99	49	10	133	42	28
	131	15	19	99	52	—	135	41	11
	135	33	28	117	59	16	137	30	6
	137	42	5	117	62	—	139	24	10
	137	52	10	139	43	10	—	—	—
	—	—	—	139	47	—	—	—	—
	144	18	26	142	47	10	—	—	—
	144	26	19	142	45	—	—	—	—
	144	38	Nil	—	—	—	—	—	—
1·0	—	—	—	99	55	7	133	49	19
	—	—	—	117	61	8	135	32	12
	—	—	—	117	—	9	137	47	10
	—	—	—	139	37	6	139	24	13
	—	—	—	139	46	—	—	—	—
	—	—	—	142	39	10	—	—	—
	—	—	—	142	50	—	—	—	—

acid and amide does not give a true idea of the hydrolysis of the carbamyl group, unless the fact that some of the acid is excreted as glucuronide is taken into account (cf. Table 3).

The nature of the ether-soluble acid was investigated in the case of *p*-aminobenzoic acid. The ether extract from the urine of six rabbits which had each received 2 g. of the acid yielded 4 g. pure *p*-aminobenzoic acid. No other metabolite, e.g. *p*-aminohippuric acid, was detected. Owing to the difficulty of obtaining and estimating *o*- and *m*-aminohippuric acids the ether-soluble acid from the *o*- and *m*-isomers was not examined. Quick (1932) was unable to devise any satisfactory procedure for estimation or identification of aminohippuric acids in urine. It is theoretically possible that the apparently low results for anthranilic acid, at 0·5 g./kg. dose level, may be due to the formation of a conjugate which is not estimated by the method used.

Diazotizable material. Table 2 shows the results obtained for excretion of diazotizable material after administration of the three aminobenzoic acids and their amides. The values obtained directly on the urine, and given under the heading 'free', represent the sum of free acid or amide plus acid conjugated as ester-type glucuronide, if present; other substances which might contribute are conjugates of the acids with glycine (aminohippuric acids) or *O*-conjugates of aminohydroxybenzoic acids or amides with sulphuric or glucuronic acids formed as a result of oxidation. The aminohydroxybenzoic acids which have been identified as oxidation products (and presumably their amides) do not diazotize and couple under the conditions of estimation. Whilst it is not definitely established that the *O*-conjugates diazotize and couple, it is possible that they do. We find, for example, that *o*-aminophenylglucuronide diazotizes and couples, although *o*-aminophenol itself does not. Available evidence (see Table 5) suggests that the contribution of *O*-conjugates to the total of 'free' diazotizable material would be of significant magnitude only in the case of *o*-aminobenzamide. The additional diazotizable material obtained after hydrolysis of the urine is given under the heading 'acetyl' and can be assumed to represent acetamidobenzoic acid or amide except in the case of *o*-aminobenzamide. Here hydrolysis of an *O*-conjugate could cause a decrease in diazo value large enough to mask an increase due to deacetylation. We actually found that the hydrolyzed values never exceeded the unhydrolyzed, but since we were unable to isolate any *o*-acetamidobenzamide we have recorded 'Nil' values in Table 2.

The fact that neither anthranilic acid nor its amide appear to be acetylated in the rabbit may account for the greater toxicity of these compounds as compared with the other isomers. The values obtained for the percentage of 'free' amide excreted are certainly high since some amide is hydrolyzed to acid which gives a more intense colour on diazotization and coupling. It is not possible to calculate the true values with accuracy without knowing whether *o*-aminohippuric acid is present, the full extent of amide hydrolysis (i.e. whether any *o*-aminobenzoylglucuronide has been formed), and the diazotization and coupling properties of the *O*-conjugates. Table 4 shows that the sulphate conjugation of *o*-aminobenzamide corresponds to 26–40% of the dose. This is presumably due to 3- and 5-hydroxyanthranilic acids or their amides. A further small amount of these compounds may be excreted as ether glucuronide.

In the case of *m*-aminobenzamide there is a clear indication that acetylation occurs. The values for free amide excreted can be taken as reasonably accurate since the difference in colour intensity of the diazotized and coupled

Table 2. *Excretion of diazotizable material, free and acetylated (calculated as aminobenzoic acid or amide) by rabbits after administration of aminobenzoic acids and amides*

Dose (g./kg.)	Percentage of dose excreted as diazotizable material														
	o-Compound					*m*-Compound					*p*-Compound				
	Rabbit no.	Acid		Amide		Rabbit no.	Acid		Amide		Rabbit no.	Acid		Amide	
		Free	Acetyl	Free	Acetyl		Free	Acetyl	Free	Acetyl		Free	Acetyl	Free	Acetyl
0·25	131	75	Nil	33	Nil	—	—	—	—	—	103	87	9	22	46
	131	67	Nil	—	—	—	—	—	—	—	103	62	9	25	76
	135	81	Nil	41	Nil	—	—	—	—	—	103	77	4	—	—
	137	69	—	36	Nil	—	—	—	—	—	113	81	21	20	64
	144	59	Nil	31	Nil	—	—	—	—	—	113	80	13	16	68
	—	—	—	—	—	—	—	—	—	—	113	—	—	15	73
	—	—	—	—	—	—	—	—	—	—	121	83	9	24	81
	—	—	—	—	—	—	—	—	—	—	121	67	14	23	78
	—	—	—	—	—	—	—	—	—	—	121	—	—	21	77
	—	—	—	—	—	—	—	—	—	—	125	73	16	19	60
	—	—	—	—	—	—	—	—	—	—	125	67	4	—	—
	—	—	—	—	—	—	—	—	—	—	133	—	—	14	81
	—	—	—	—	—	—	—	—	—	—	137	—	—	12	62
0·5	131	69	Nil	50	Nil	99	70	10	23	41	103	87	1	37	45
	131	—	—	45	Nil	99	85	12	27	35	103	67	15	28	85
	135	68	Nil	48	—	99	82	23	—	—	113	71	18	35	51
	137	75	Nil	48	—	99	92	9	—	—	113	75	2	28	45
	144	71	Nil	45	Nil	117	78	Nil	24	42	113	78	14	—	—
	144	67	Nil	39	Nil	117	80	14	—	—	121	62	7	28	58
	—	—	—	—	—	117	76	19	—	—	121	83	21	31	55
	—	—	—	—	—	139	83	13	52	10	121	72	14	—	—
	—	—	—	—	—	139	100	Nil	50	10	125	70	13	38	41
	—	—	—	—	—	139	94	4	—	—	125	76	17	—	—
	—	—	—	—	—	142	74	11	50	6	—	—	—	—	—
	—	—	—	—	—	142	86	7	52	9	—	—	—	—	—
	—	—	—	—	—	142	82	4	54	2	—	—	—	—	—
	—	—	—	—	—	142	—	—	37	14	—	—	—	—	—
1·0	—	—	—	—	—	99	96	4	38	42	103	44	16	36	24
	—	—	—	—	—	99	—	—	41	30	103	57	1	—	—
	—	—	—	—	—	117	90	9	30	43	103	49	Nil	—	—
	—	—	—	—	—	117	81	17	25	41	113	51	1	42	37
	—	—	—	—	—	139	100	Nil	66	6	113	41	3	—	—
	—	—	—	—	—	142	70	Nil	49	6	121	41	3	34	35
	—	—	—	—	—	142	79	2	48	5	121	53	2	—	—
	—	—	—	—	—	—	—	—	—	—	121	62	1	—	—
	—	—	—	—	—	—	—	—	—	—	125	61	5	39	38
	—	—	—	—	—	—	—	—	—	—	125	86	8	—	—

acid and amide is of the same order as the experimental error of the diazo method. As with the *o*-isomer, the metabolite formed by oxidation would probably not contribute to the diazotizable material. The amounts formed, however, are much smaller and will be of correspondingly less significance. The *m*-acid is acetylated to only a small extent. The oxidation product of *p*-aminobenzamide would also probably not contribute to the diazotizable material.

Table 2 also shows that different rabbits may have widely different powers of acetylation. Rabbits 139 and 142 are consistently poor acetylators compared with 135 and 137. It has been our experience that rabbits which are not good acetylators are much less resistant to toxic amino compounds.

Reducing substances. The average base line values were 209 mg./day on bran, oats and cabbage and 185 mg. on the pellet diet. The results obtained after administration of aminobenzoic acids are given in Table 3. It will be seen that there is evidence of conjugation for all three isomers. The glucuronide excretion after administration of amides could be reliably estimated only in the case of the *p*-amide which is oxidized to only a very slight extent. The formation of considerable amounts of reducing oxidation products from the *o*- and *m*-isomers invalidates the glucuronide estimation.

Ethereal sulphate. The average base line values for SO_3 were 21 mg./day on the bran, oats and cabbage diet and 41 mg. on the pellet diet. The results obtained after administration of aminobenzoic acids and amides are summarized in Table 4. Increases in ethereal sulphate excretion were observed only after giving the amides. We did not confirm the observation of Mitsuba & Ichihara (1927) that anthranilic acid causes an increase in ethereal sulphate excretion.

Table 3. *Excretion of reducing substances (calculated as glucuronic acid) by rabbits after administration of aminobenzoic acids and amides*

	Percentage of dose excreted as glucuronide (ester or ether type)							
	o-Compound		*m*-Compound		*p*-Compound			
							Amide	
Dose (g./kg.)	Rabbit no.	Acid (ester type)	Rabbit no.	Acid (ester type)	Rabbit no.	Acid (ester type)	(Ester type)	(Ether type)
0·25	131	44	—	—	103	—	0	0
	131	65	—	—	113	8	0	0
	144	43	—	—	113	24	—	—
	144	47	—	—	121	—	8	0
	—	—	—	—	125	—	6	0
0·5	131	54	99	4	103	19	9	0
	144	43	99	17	113	22	10	1
	144	62	117	7	121	30	1	1
	—	—	117	8	125	14	3	0
	—	—	117	8	125	13	—	—
	—	—	139	4	—	—	—	—
	—	—	142	7	—	—	—	—
1·0	—	—	99	6	103	—	6	0
	—	—	99	8	113	21	7	0
	—	—	117	6	121	14	3	0
	—	—	117	7	125	15	6	0
	—	—	117	7	—	—	—	—
	—	—	139	7	—	—	—	—
	—	—	139	8	—	—	—	—
	—	—	142	6	—	—	—	—
	—	—	142	7	—	—	—	—
	—	—	142	10	—	—	—	—

Table 4. *Excretion of ethereal sulphate by rabbits after administration of aminobenzoic acids and amides*

	Percentage of dose excreted as ethereal sulphate								
	o-Compound			*m*-Compound			*p*-Compound		
Dose (g./kg.)	Rabbit no.	Acid	Amide	Rabbit no.	Acid	Amide	Rabbit no.	Acid	Amide
0·1	—	—	—	—	—	—	103	0	—
	—	—	—	—	—	—	113	0	—
0·25	131	0	26	—	—	—	103	—	2
	144	0	32	—	—	—	113	0	—
	—	—	—	—	—	—	121	0	—
0·5	131	0	33	—	—	—	125	0	2
	144	0	40	99	0	—	121	—	1
1·0	—	—	—	99	0	6	103	—	5
	—	—	—	99	0	7	113	—	2
	—	—	—	117	0	5	121	0	1
	—	—	—	142	0	7	125	—	1

Qualitative studies

Isolation of m- *and* p-*acetamidobenzamides*

In the case of *m*- and *p*-aminobenzamides, the *N*-acetyl derivatives could be isolated by continuous extraction with ether from the urine of rabbits to which the free amides had been administered by stomach tube (dose level 1 g./kg.). The material thus obtained was dissolved in water, treated with charcoal and recrystallized from water. The products obtained were identical with authentic specimens of *m*-acetamidobenzamide (m.p. 221°) and *p*-acetamidobenzamide (m.p. 274°), which were prepared by the action of acetic anhydride on the respective amides. This is direct evidence that in these compounds the carbamyl group is not completely hydrolyzed in the rabbit. Similar evidence in the case of *o*-aminobenzamide is provided by the fact that, although the acetyl derivative could not be isolated by ether extraction, the amide itself was. Table 2 shows that this amide is not acetylated *in vivo*.

Isolation of ester type glucuronides as metabolites of acids

The method of isolation was essentially as previously described (Bray *et al.* 1947). Since the compounds being isolated were ester glucuronides and consequently less stable than those of the ether type, the time taken during isolation was reduced to a minimum.

Table 5. *Excretion of metabolites of aminobenzoic acids and amides by the rabbit*

				Percentage of dose excreted as				
Dose (g./kg.)	Isomer	Compound	Ether soluble acid	Ester glucuronide	Free NH_2 derivative	Acetyl NH_2 derivative	Ethereal sulphate	Estimated average hydrolysis (%)
0·25	Ortho	Acid	60–79	43–65	59–81	0	0	33*
		Amide	27–38	—	31–41	0	26–32	
0·5		Acid	15–52	43–62	67–75	0	0	12*
		Amide	0–28	—	39–50	0	33–40	
0·5	Meta	Acid	43–62	4–17	70–100	0–23	0	12*
		Amide	10–16	—	23–52	2–42	—	
1·0		Acid	37–61	6–10	70–100	0–17	0	8*
		Amide	6–10	—	25–66	5–43	5–7	
0·25	Para	Acid	20–45	8–24	62–87	4–21	0	15
		Amide	0–29	0–8	12–25	46–81	2	
0·5		Acid	24–42	13–30	62–87	4–21	0	20
		Amide	6–28	1–10	28–38	41–85	1–2	
1·0		Acid	24–49	14–21	44–86	0–16	0	21
		Amide	10–19	3–7	34–42	24–38	1–5	

* These values are probably low, since no values are available for ester glucuronide.

p-*Aminobenzoylglucuronide.* The solution remaining after the removal of PbS was evaporated to small bulk at 50–60° under reduced pressure. On standing crystallization occurred. The red-brown solid was filtered off, dissolved in water, decolorized with charcoal and recrystallized from aqueous ethanol. The product formed white crystalline flakes (m.p. 193° with decomp.; $[\alpha]_D^{24^\circ}$ $-5{\cdot}5^\circ$ in water). It strongly reduced Benedict's and Fehling's solutions, and appeared to be a *complex* of equimolecular proportions of *p*-aminobenzoic acid and *p*-aminobenzoylglucuronide. (Found: C, 52·7; H, 4·41; N, 5·82%; equiv. (by titration) 225; NH_2 (diazotizable), 6·45%; glucuronic acid, 40·5%. $C_{20}H_{22}O_{10}N_2$ requires C, 53·3; H, 4·88; N, 6·23%; equiv., 225; NH_2, 7·12%; glucuronic acid, 43·2%.) The yield was 10% of the dose.

Low values for glucuronic acid content were consistently obtained by the Shaffer-Hartmann method. This is probably due to the great lability of these compounds to heat. Pure preparations on warming even slightly, whether dry or in solution, rapidly developed a bright-red colour.

By continuous ether extraction of an aqueous solution of the complex, *p*-aminobenzoic acid was isolated and characterized. By careful evaporation of the remaining solution at a low temperature under reduced pressure, a substance crystallizing as fine white needles was obtained which, after recrystallization from aqueous ethanol, melted (with decomp.) at 208–210°. It was strongly reducing and showed $[\alpha]_D^{24^\circ}$ -12° (in water). It appeared to be p-*aminobenzoylglucuronide.* (Found: C, 49·6; H, 4·34; N, 4·78%; equiv. (by titration), 321; NH_2 (diazotizable), 5·05%; glucuronic acid, 54·2% (see above). $C_{13}H_{15}O_8N$ requires C, 49·8; H, 4·8; N, 4·47%; equiv., 313; NH_2, 5·12%; glucuronic acid, 62·1%.)

Further evidence that the compound first isolated was a complex of *p*-aminobenzoylglucuronide and *p*-aminobenzoic acid is provided by the fact that, when products were isolated which were syrups and could not be induced to crystallize directly, in most cases addition of *p*-aminobenzoic acid resulted in the crystallization of the complex.

o-*Aminobenzoylglucuronide.* The method described above gave a readily soluble syrup which could not be crystallized. It did not form a crystalline complex with anthranilic acid or with *p*-aminobenzoic acid nor has any crystalline derivative yet been obtained.

We were unable to isolate a glucuronide from *m*-aminobenzoic acid. Table 3 shows that this isomer is conjugated with glucuronic acid to a very much smaller extent than the others.

Isolation of oxidation products from o-*aminobenzamide*

This compound (30 g.) was administered to 20 rabbits and 24 hr. urine (8 l.) collected. This was hydrolyzed by boiling with conc. HCl (1/5 vol.) for 1 hr., made slightly acid to litmus and extracted continuously with ether for 48 hr., until the blue fluorescence was no longer visible in the ether layer. Two methods were attempted for the isolation of oxidation products from the ether-soluble material.

Chromatography. A solution of the ether-soluble material in ethanol and benzene was poured through an alumina column and anthranilic acid eluted with the same solvent mixture. Anthranilic acid could be readily distinguished from other metabolites in the column owing to its very strong fluorescence in ultraviolet light. It was, however, impossible to elute either with boiling water or organic solvents all the phenolic material which could be shown, by means of the $FeCl_3$ reaction, to remain on the column.

Fractional crystallization. The ether-soluble material was isolated by evaporation of the extract to dryness *in vacuo* and dissolved in 50% (v/v) aqueous ethanol. By progressive concentration under reduced pressure two main solid fractions were isolated and recrystallized from 60% (v/v) aqueous ethanol. The compounds isolated in all these experiments showed a great tendency to darken in air, especially in solution. The addition of a few drops of SO_2 water prevented this and enabled almost colourless products to be obtained.

The fractions obtained were as follows:

(1) Pale brown needles (yield 150 mg. from solid material extracted from 3 l. urine), soluble in ethanol only with difficulty. Purification was effected by continuous extraction with ether from an aqueous suspension. Ethereal solutions showed an intense blue-violet fluorescence in ordinary light. This compound darkened on heating and decomposed at 248°. It gave a brown coloration with $FeCl_3$, a positive indophenol reaction and reduced aqueous $AgNO_3$ solution in the cold. It gave no yellow coloration with HNO_2 and diazotized solutions did not couple normally with dimethyl-α-naphthylamine or with *N*-(1-naphthyl)-ethylenediamine dihydrochloride, but in concentrated solutions (see p. 435) a violet colour, pale in the case of the first coupling agent and intense with the second, developed slowly, reaching maximum intensity in about 1 hr. With Br_2 water it gave a red-brown coloration. (Found: C, 54·8; H, 4·74; N, 9·2; calc. for $C_7H_7O_3N$; C, 54·8; H, 4·58; N, 9·14%.) The compound was identical in all respects with synthetic 5-hydroxyanthranilic acid prepared by reductive fission of 4-hydroxy-2-carboxyazobenzene in alkaline solution by means of $Na_2S_2O_4$ (cf. Limpricht, 1891). (Found: C, 54·6; H, 4·67; N, 9·09%; m.p. 248° (decomp.); Limpricht gives 235°; Bamberger & Pyman (1909) give 246–252° depending upon rate of heating.)

The method of preparation of the azo compound fixes only the relative position of the methyl and hydroxyl groups. The position of the amino group was established by conversion of the hydrochloride of the acid to *p*-aminophenol by sublimation *in vacuo*. This was characterized both as such and as its *N*-acetyl derivative. Both the synthetic and biosynthetic specimens of 5-hydroxyanthranilic acid were acetylated by treatment with acetic anhydride at ordinary temperature and in both cases crystalline products were obtained which melted, either alone or mixed together, at 238–239°. The compounds were shown to be monoacetyl derivatives inasmuch as they gave slight brown colorations with $FeCl_3$ and contained 6·62 and 6·95% N, respectively. Calc. for $C_9H_9O_4N$, 7·18%. Friedländer (1916) gives m.p. 227° and Kalle and Co. (1911), 224–226° (decomp.)

(2) This fraction on recrystallization yielded two compounds (*a*) and (*b*):

(*a*) Pale brown needles (yield 50–60 mg.), darkening at 230–240° and decomposing at 260°. This compound gave a dark brown coloration with $FeCl_3$, no indophenol reaction, no diazo reaction but an intense yellow colour with HNO_2. It was slightly reducing to aqueous $AgNO_3$ and gave no coloration with bromine water. In ethereal solution it showed a mauve fluorescence in ordinary light. The properties of this compound were identical with those of synthetic 3-hydroxyanthranilic acid. The only previous record of a compound to which this structure is attributed is due to Keller (1908), who prepared it by the action of red P and HI on damascenine, an alkaloid from *Nigella damascena*. The melting point is stated to be 163–164°, while our synthetic compound decomposed at 260°. Keller did not confirm the structure of his compound by synthesis, but the properties of our compound and its method of preparation suggest that it is, in fact, 3-hydroxyanthranilic acid. *m*-Acetamidobenzoic acid was nitrated as described by Kaiser (1885) to give 2-nitro-3-acetamidobenzoic acid, isolated in 20% yield as pale yellow needles, melting at 245° (Kaiser gives 240–241°). This compound was deacetylated and deaminated by boiling for about 1 hr. with aqueous KOH (25%) until the evolution of NH_3 ceased (cf. Griess, 1878), the 2-nitro-3-hydroxybenzoic acid formed being isolated in 70–80% yield, m.p. 162–163° (Griess found 178°). This compound was reduced with $SnCl_2$ and HCl by the method of Zahn (1900) and *3-hydroxyanthranilic acid* obtained as a slightly pink crystalline powder, darkening on heating to 240° and decomposing at 260°. It was a derivative of *o*-aminophenol inasmuch as its hydrochloride on sublimation *in vacuo* yielded this compound and since it gave no diazo reaction, but gave the typical yellow diazo-oxide colour with HNO_2. Further evidence of the configuration is provided by the fact that it, or its amide, is a metabolite of *o*-aminobenzamide. It contained 8·99% N; $C_7H_7O_3N$ requires 9·14%. The identity of the compound isolated from urine with this synthetic compound was confirmed by preparing an acetyl derivative by the action of acetic anhydride at ordinary temperature, and comparing it with the acetyl derivative of the synthetic compound, prepared in the same way. This compound does not appear to have been prepared before. Both the synthetic acetate and the acetate of the biosynthetic acid melted at 143–145°, either alone or mixed together. Both gave a slight dark brown coloration with $FeCl_3$, which suggests that the compound was the monoacetyl derivative. This was confirmed by the analysis of the synthetic specimen. It contained 7·14% N; $C_9H_9O_4N$ requires 7·18%.

(*b*) Pale brown needles (yield 20 mg.), decomposing at 230°. This compound was fairly soluble in aqueous ethanol, from which it was recrystallized, and gave no coloration with $FeCl_3$, negative indophenol and diazo reactions, a slight yellow coloration with HNO_2. Analysis gave C, 52·3; H, 4·62; N, 14·2%. The nature of this compound has not been determined. The analysis suggests that it might be an impure specimen of an aminohydroxyhippuric acid ($C_9H_{10}O_4N_2$ requires C, 51·4; H, 4·76; N, 13·34%), but it has not been possible to investigate it further.

The mother liquors from which these compounds were obtained were combined, evaporated to dryness *in vacuo* and chromatographed on alumina (see above). After elution of anthranilic acid, the presence of phenolic compounds on the column could be shown. Elution with boiling water enabled a further small amount of 5-hydroxyanthranilic acid to be isolated.

Isolation of oxidation products from m-*aminobenzamide*

To each of 19 rabbits 2 g. *m*-aminobenzamide were given, and the urine passed in the following 24 hr. was collected and hydrolyzed by boiling for 1 hr. with 1/5 vol. conc. HCl. The hydrolyzed urine was continuously extracted with ether for 48 hr., first at pH 6·5 and then at pH 5·5. The extracts obtained in this way had very similar properties and were recombined (extract *A*). The urine was then evaporated *in vacuo* to one-quarter bulk and again extracted with ether for 48 hr. (extract *B*). It was finally evaporated to dryness *in vacuo* at 60–70° and the residue extracted with 96% ethanol (extract *C*).

Extract *A* was evaporated to dryness, dissolved in water, treated with charcoal and progressively concentrated to yield various crystalline fractions and a syrupy residue (*D*). The crystalline fractions had indefinite melting points ranging from 165 to 195° and all gave positive diazo reactions and a brownish red colour with $FeCl_3$. They were therefore recombined and recrystallized from 96% ethanol. Part of the material (500 mg.) did not dissolve easily. It contained some ash and was purified by extraction with ether from an acidified aqueous solution, followed by recrystallization from water. Yield 20 mg. of mauve needles, decomposing at 253°, which gave a purple coloration with $FeCl_3$, a negative diazo reaction, no coloration with HNO_2, a reddish brown coloration and precipitate with Br_2 water, a positive indophenol reaction, and reduced ammoniacal $AgNO_3$. These properties are those of 5-aminosalicylic acid. Recrystallization of the material which was soluble in 96% ethanol (above) yielded as a first fraction 30 mg. of white crystals whose properties, apart from colour, were the same as those of the compound just described. The identity of these two specimens with 5-aminosalicylic acid was confirmed by conversion into the *NO*-diacetyl derivative, m.p. 192°, which did not depress the melting point (194°) of an authentic specimen of 5-acetamidoacetylsalicylic acid. Meldola, Foster & Brightman (1917) give m.p. 283° for the acid and 197° for the acetate (actually the diacetate; present authors unpublished). The remainder of the ethanol-soluble material (6 g.) consisted of *m*-aminobenzoic acid. The syrupy residue *D* was treated with acetic anhydride, dissolved in water and fractionated. Three fractions were obtained: (1) *m*-acetamidobenzoic acid (1 g.); (2) a crystalline fraction of lower melting point (1 g.) which on deacetylation and fractionation with ethanol yielded mainly *m*-aminobenzoic acid; (3) a syrupy residue (2 g.) which was chromatographed on alumina, using ethanol-benzene as solvent. The fractions thus obtained after deacetylation gave a purple-brown coloration with $FeCl_3$ and a slight yellow colour with HNO_2, but contained relatively large amounts of *m*-aminobenzoic acid which prevented the isolation of the phenolic compound present.

Extract *B*, treated in the same way as extract *A*, yielded three main fractions: (1) white crystals (40 mg.) giving a purple coloration with $FeCl_3$, a positive diazo reaction, developing slowly, no yellow colour with HNO_2 and a bright-green colour and precipitate with Br_2 water. Analysis gave: C, 49·2; H, 5·1; N, 17·7%. This compound was neither an acid nor an ammonium salt. It did not melt, but partially sublimed with decomposition at 310°. Its identity has not yet been established, but it is suggested that it might be a hydrated amide of an aminohydroxybenzoic acid. $C_7H_8N_2O_2.H_2O$ requires C, 49·4; H, 5·9; N, 16·5%; (2) white needles (200 mg.) which did not melt below 300° and contained an appreciable amount of ash; 150 mg. were acetylated and gave a compound which was proved to be 5-acetamidosalicylic acid. Sublimation *in vacuo* of the remainder yielded crystalline *p*-aminophenol, presumably derived from 5-aminosalicylic acid by decarboxylation; (3) a syrup, which on chromatography (as above) yielded 10 mg. crystals and a syrupy fraction. After recrystallization from water the crystals melted at 215° (decomp.) and gave a yellow colour with HNO_2, but a negative diazo reaction. With $FeCl_3$ they gave a bluish purple coloration. It is probable that this compound was 3-amino-4-hydroxybenzoic acid, but, owing to the small amount isolated, it was not possible to characterize it finally. The syrup gave a purple-brown coloration with $FeCl_3$, but contained also an appreciable amount of *m*-aminobenzoic acid which prevented isolation of the substance responsible.

Extract *C* gave a purple colour with $FeCl_3$, but no metabolite of *m*-aminobenzamide could be isolated.

Isolation of oxidation products from p-*aminobenzamide*

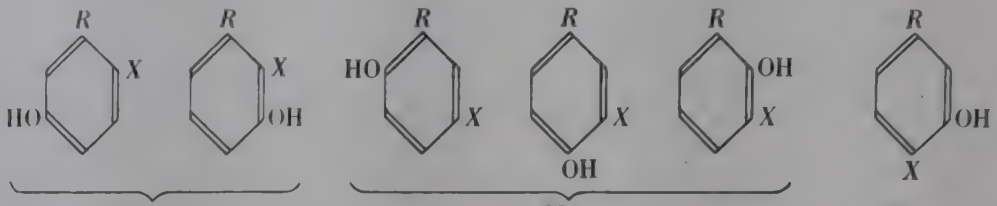
p-Aminobenzamide (30 g.) was fed to 12 rabbits and 24 hr. urine collected. This was hydrolyzed, neutralized and extracted as in the case of *o*-aminobenzamide. The ether-soluble material was recrystallized from aqueous ethanol and a considerable amount of *p*-aminobenzoic acid recovered. The mother liquors gave a bluish coloration with $FeCl_3$ and ultimately yielded about 15 mg. of a white crystalline material which gave a dark-blue coloration with $FeCl_3$, a negative diazo reaction and an intense yellow coloration with HNO_2. It decomposed at 225–227°. These properties are identical with those of synthetic 4-amino-3-hydroxybenzoic acid, prepared by the nitration of *m*-hydroxybenzoic acid (Griess, 1887) and reduction of the resulting 4-nitro-3-hydroxybenzoic acid with $SnCl_2$ and HCl (cf. Zahn, 1900). M.p. 226° (Einhorn & Pfyl (1900) give 216°). The identity of the synthetic and biosynthetic acids was confirmed by comparing their *acetyl* derivatives. That of the compound from urine melted (with decomposition) at 238–239°, that of the synthetic compound at 242–244° and a mixture of the two at 240–241°. This acetate does not appear to have been prepared previously.

It should be noted that although these oxidation products were isolated and characterized as acids, it is probable that they are excreted as amides (see p. 434). It is reasonable to conclude that the introduction of the hydroxyl group takes place while the carbamyl group is intact, since the acids themselves give rise to no increased excretion of ethereal sulphate. Whether or not hydrolysis of the carbamyl group takes place more readily in aminohydroxybenzamides than in aminobenzamides is not yet known.

DISCUSSION

The results given in Tables 1–4 and summarized in Table 5 show clearly that there is a considerable difference between the metabolism of the aminobenzoic acids and their amides. Differences in oxidation (as shown by the increase in ethereal sulphate excretion) and in acetylation provide indirect evidence of the comparative stability of the amide grouping in these compounds *in vivo*, while the isolation of unchanged *o*-aminobenzamide, and of *N*-acetyl derivatives of the *m*- and *p*-isomers from the urine of rabbits which had received the amides indicates directly that hydrolysis of the carbamyl group is not complete. Estimations of ether-soluble acid indicate that the group is hydrolyzed to an extent of about 12–33% in the *o*-, 8–12% in the *m*- and 15–21% in the *p*-amide. The corresponding percentages of hydrolysis obtained *in vitro* with liver extracts were 9, 7 and 13 (Bray *et al.* 1948).

The identity of the oxidation products isolated is of particular interest inasmuch as the generalization suggested in a previous paper (Bray *et al.* 1947) can now be more clearly defined. Taking into account the work of other investigators and work in this laboratory, it appears to be a general rule that, in the hydroxylation *in vivo* of a disubstituted aromatic compound, $R.C_6H_4.X$, where R is COOH, $CONH_2$, SO_2NH_2 or CH_3 and X is OH or NH_2 ($NHCOCH_3$ in acetotoluidides), the position of the hydroxyl group introduced is, as might be expected on purely chemical grounds, either para or ortho to X, giving, when R and X are themselves ortho, I or II; when R and X are meta, III or IV (V has not yet been reported) and, when R and X are para, VI.



I II III IV V VI

It has been proved in the present investigation that in the case of *o*-aminobenzamide the two possible oxidation products, I and II, are both formed. Evidence for the formation of the two expected oxidation products of orthanilamide has been put forward by Stubbs & Williams (1947). From *m*-aminobenzamide the main oxidation product isolated corresponded to III. There was, however, evidence of another oxidation product which was probably IV. From *p*-aminobenzamide the only oxidation product isolated was, as would be expected, VI.

The case of the acetotoluidides is of interest since we have shown (unpublished result) that in the rabbit the *o*-isomer gives rise mainly to 2-amino-5-hydroxytoluene (6-amino-*m*-cresol) (I), excreted both as glucuronide and ethereal sulphate, whereas Jaffe & Hilbert (1888) found that the dog excreted a benzoxazolone derivative, presumably derived from 2-amino-3-hydroxytoluene (2-amino-*m*-cresol) (II). We cannot yet say whether this isomer is also formed by the rabbit. In the *m*- and *p*-acetotoluidides we have confirmed Jaffe & Hilbert's (1888) observation that the oxidation of the methyl group takes précedence, aminobenzoic acids appearing as the main excretion products. Phenolic compounds are, however, formed in smaller amounts, particularly with the *m*-isomer, but have not yet been identified. All these compounds are actually excreted as *N*-acetyl derivatives, since only a small amount of deacetylation takes place *in vivo*.

It would be expected that in the compounds under discussion the group X (OH or NH_2) will determine the position of the entering hydroxyl group, group R having no significant directing effect. The hydroxylated compounds, in fact, correspond to those formed when the group R is a hydrogen atom, i.e. in monosubstituted compounds. Phenol is converted in the animal to catechol and hydroquinone (Baumann & Preusse, 1879) and aniline (as acetanilide or formanilide) to *o*-aminophenol (as benzoxazolone) in the dog and to *p*-aminophenol in the rabbit (Jaffe & Hilbert, 1888; Kleine, 1896–7). Similar results have been observed if group X is modified, e.g. phenetole is converted to monoethyl hydroquinone in the dog (Kossel, 1880); β-phenylhydroxylamine and cupferron (ammonium nitrosophenylhydroxylamine) to *p*-aminophenol in the rabbit (Sieburg, 1914) and dimethylaniline to *o*-aminophenol in the dog and to *p*-monomethylaminophenol in the rabbit (Horn, 1936*a*, *b*). We have also shown (unpublished result) that phenylurea is converted in the rabbit to *p*-hydroxyphenylurea, which is excreted mainly as glucuronide. So far as we are aware, compounds in which the group X and a hydroxyl group occupy positions meta to one another have never been found as products of mammalian metabolism.

SUMMARY

1. The metabolism of *o*-, *m*- and *p*-aminobenzoic acids and their amides in the rabbit has been studied both qualitatively and quantitatively.

2. There is evidence that the carbamyl group is hydrolyzed in the rabbit to an extent of 12–33% in the *o*-, 8–12% in the *m*-, and 15–21% in the *p*-compound.

3. Among the compounds isolated were *m*-acetamidobenzamide and *p*-acetamidobenzamide as metabolites of the amides themselves and *p*-aminobenzoylglucuronide as a metabolite of *p*-aminobenzoic acid. This glucuronide forms a crystalline complex with *p*-aminobenzoic acid.

4. Four aminohydroxybenzoic acids have been isolated from the hydrolyzed urines of rabbits which had received the aminobenzamides under investigation. In this way 3- and 5-hydroxyanthranilic acids were obtained from *o*-aminobenzamide, 3-amino-6-hydroxybenzoic acid from *m*-aminobenzamide and 4-amino-3-hydroxybenzoic acid from *p*-aminobenzamide.

5. This type of biological hydroxylation is discussed from the point of view of the position taken up by the hydroxyl group introduced with respect to the other groups present.

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Separation of Acidic Amino-acids by means of a Synthetic Anion Exchange Resin

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Synthetic ion-exchange resins have recently been widely used for the separation of basic and acidic from neutral compounds. Thus Cannan (1944), by repeated applications of Amberlite IR4, separated glutamic and aspartic acids quantitatively from protein hydrolysates. Earlier, Freudenberg, Walch & Molter (1942) had used a column of Wolfatite M for the separation of the dicarboxylic amino-acids, and Cleaver, Hardy & Cassidy (1945) investigated the chromatographic separation of amino-acids by organic exchange resins. Recently, Tiselius, Drake & Hagdahl (1947) have employed synthetic ion exchange resins chromatographically for the quantitative separation of amino-acids into groups. The aim of the present investigation was to extend such methods to cover the separation of the acidic amino-acids from one another (at pH 2·5) after their isolation from the rest of the protein hydrolysate (at pH 3–4). Although completely quantitative recoveries were not obtained, complete separations have been achieved. The separation depends on differences in net charge between the acidic amino-acids at a given pH (Fig. 1). Thus, glutamic acid with pK_1 2·16 and pK_2 4·32 (Neuberger, 1936) has a net charge of −0·31 at pH 4; aspartic acid with pK_1 1·88 and pK_2 3·65 (Miyamoto & Schmidt, 1931) has a net charge of −0·69 at pH 4; whilst cysteic acid with pK_1 1·88 and pK_2 1·30 (Andrews & Schmidt, 1927) has a net charge of −0·99 at this pH. It would therefore be expected that a column of Amberlite IR 4 maintained uniformly at pH 4, because of its positive charge, will preferentially retain cysteic, aspartic and glutamic acids in this order. Theoretically, it would appear that the optimum pH for the separation of glutamic from aspartic acid is about 4, since at this pH the largest difference in net charge occurs, but experimentally it was found that for satisfactory separation a pH of 2·5 was necessary. At this pH glutamic and aspartic acids normally carry a net positive charge of +0·29 and +0·13 respectively. Nevertheless, at this pH they are retained on Amberlite IR 4 columns and can be separated by elution in relatively small volumes; at higher pH values, although separation will take place, relatively large volumes are required. This suggests that the pK values are depressed when these amino-acids are adsorbed on Amberlite IR 4. Similarly, cysteic acid is partially retained at pH 1·5, although its net charge at this pH should be almost zero.

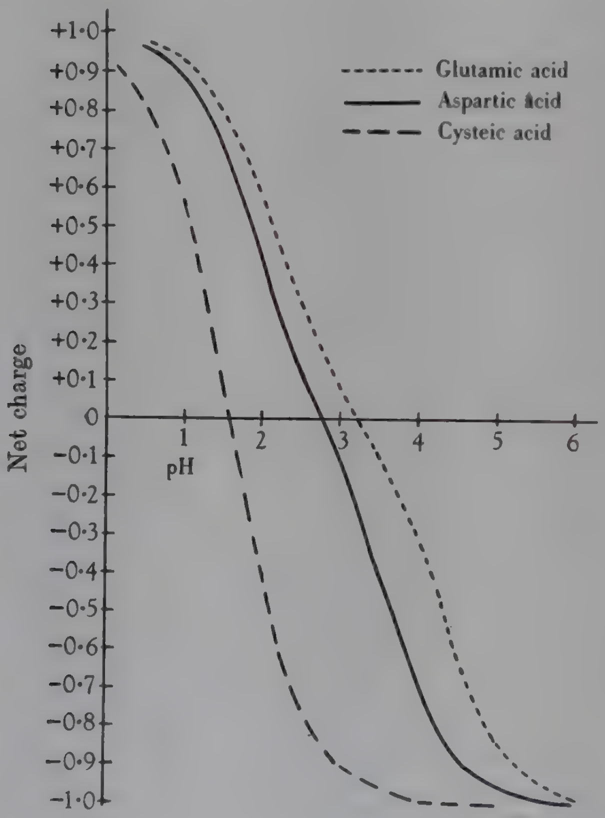

Fig. 1. Net charge of glutamic, aspartic and cysteic acids at different pH values, calculated from the dissociation constants given in the text.

To apply these principles, care has been taken to bring the Amberlite to the required pH and to maintain it uniformly in that part of the column where separations are taking place. In order to establish equilibrium conditions, the resin was finely powdered and the width of the column kept small in relation to its height so that low rates of flow were maintained. In the first stage, no difficulty was found in effecting the separation of the acidic from the basic and neutral amino-acids in a protein hydrolysate at pH 3–4. As is shown by its titration curve (Fig. 2), at this rather acid pH, the percentage charge on the resin is sufficiently high for it to compete successfully with lysine and arginine for the acidic amino-acids. The departure from the expected sigmoid shape of the curve below pH 1 may be due to decomposition of the resin. This is also suggested by the fact that the nitrogen (Kjeldahl) washed from a column by acid is about ten times as high for strong acid (pH 0–1) as for weak acid (pH 3–4) and is independent of the history (previous washings) of the column. By the above means and by the application of the chromatographic principle, considerable economy of resin was achieved. Separations have been followed by partition chromatography on paper (Consden, Gordon & Martin, 1944) of samples from each eluate; this method has provided a convenient means for identifying the amino-acids present in each fraction, without using more than negligible quantities.

The copper titration method of Pope & Stevens (1939) was chosen as a convenient means of estimating the acidic amino-acids after separation by the resin and was adapted to deal with micro quantities. Unfortunately, it was not found possible to obtain fully quantitative recoveries of glutamic and aspartic acids after their separation from synthetic mixtures. The recovery of glutamic acid was about 91 %, that for aspartic about 96 %. A possible cause for low recoveries may be formaldehyde which appears to be continually liberated even after the washing cycle employed by Cannan (1944). Attempted removal of formaldehyde failed to improve the results. Estimation of amino N or total N in the eluates by the Van Slyke manometric procedure or Kjeldahl method was tried and abandoned owing to the high blanks given by eluates washed off the column after the removal of amino-acids. Cysteic acid, as isolated from hydrolysates by means of Amberlite, is always accompanied by pigment having a similar acid strength. As similar pigment material from hydrolysates of wool not containing cysteic acid gives significant titrations in the Pope & Stevens (1939) procedure, estimations of cysteic acid would presumably give fictitiously high results.

As the synthetic ion exchange resins can absorb relatively large amounts of amino-acids (Englis & Fiess, 1944; Cleaver *et al.* 1945), they are suitable for isolations. Thus, cysteic acid has been isolated from a hydrolysate of chlorinated wool. This material was almost fully optically active, whereas that obtained by Lissizin (1928) from hair oxidized by alkaline permanganate was inactive.

METHODS

*Preparation of the Amberlite IR4.** The resin is alternately ground wet in a ball mill and sieved with a jet of water, −90 +200 grading being used for the columns. It is washed free from NH_3, treated with HCl until the supernatant liquor is permanently acid to thymol blue ($pH < 1$) and then washed with water. Alternate treatments with acid and water are repeated a number of times.

Preparation of columns. (i) The resin is stirred and washed by decantation with water until the supernatant liquid is less than 0·001 N with respect to alkali. The slurry is transferred to a glass tube of 0·5 cm. internal diameter, forming a column 20 cm. in height (containing about 2 g. air-dry resin), and washed overnight with water at a rate of approx. 15 ml./hr. This does not significantly raise the pH of the eluate, the acid concentration of which should be checked by titration before the next stage. (ii) The second column (height 30 cm.) is prepared from about 3 g. Amberlite and the pH is adjusted accurately to 2·5 by equilibration with dilute acid or water until the acidity of the supernatant fluid is slightly above 0·003 N. The material is then transferred to a 0·5 cm. diameter tube and washed overnight with 0·003 N-HCl. The acidity of the eluate is finally checked by titration. The columns should not be allowed to run dry and remain so for any length of time; otherwise extensive cracking may occur.

Procedure for estimation of glutamic and aspartic acids. The hydrolysate prepared with HCl and containing up to 15 mg. acidic amino-acids is repeatedly evaporated *in vacuo* by rotating manually in a warm water bath, allowed to stand overnight *in vacuo* over KOH, and quantitatively transferred in a small volume to the first column. Water is then passed down the column; the neutral and basic amino-acids are eluted in the first 25 ml. The next 5 ml. should be free from amino-acids. To test the eluates, a portion of the first 25 ml. and the whole of the second 5 ml. fraction are separately concentrated *in vacuo* to very small bulk and samples of each tested by partition chromatography on a strip of Whatman no. 4 paper using phenol-NH_3, developing for 5 hr. or more, with suitable control (cf. Consden *et al.* 1944). After development and treatment with ninhydrin, basic and neutral, but no acidic amino-acids, are found in the first eluate, whilst the second eluate should contain no amino-acids. If cystine is present, the eluate sample is oxidized by treatment with excess Br_2 water before chromatography; the cysteic acid thus produced occupies a position on the chromatogram not overlapping the positions of aspartic and glutamic acids (Consden, Gordon & Martin, 1946) if these are present.

The next stage consists in transferring the material remaining in the column to the second column. This may be done in either of two ways:

Method 1. The column is eluted with N-HCl causing the pigment to run down as a sharp band. When this has run out, the washing is stopped and the eluate repeatedly evaporated *in vacuo* and allowed to stand overnight *in vacuo* over KOH to remove excess HCl. It is then quantitatively transferred to the second column which is eluted with 0·003 N-HCl, 10 ml. eluates being collected. Each is concentrated *in vacuo* to a volume of 1–2 ml. and a sample (4 μl.) tested as described above.

Method 2. The column is washed with 3 ml. N-HCl, causing the pigment band to travel about half way down. (The eluate should be free from amino-acids as revealed by chromatography.) The column is then fixed to the top of the second column. Elution is continued with water, but the acid already added is sufficient to cause the pigment band to continue to travel down and eventually to pass into the lower column. When the pigment has travelled a few cm. into the second column, the top column is removed and elution of the lower column continued with 0·003 N-HCl. If no pigment is present, elution may be followed by the change in shade of the resin which is orange when strongly acid. Eluates are collected and tested as in method 1.

Method 1 has been found to behave more consistently than method 2; in proteins where the ratio of glutamic to aspartic acid is of the order 2 : 1, the glutamic acid is eluted from the second column in the first 20 ml., and aspartic acid in the next 30 ml., whereas in method 2 the stages at which glutamic and then aspartic acids begin to appear are rather less certain. Method 2 is quicker than method 1 but requires more attention.

Each eluate is evaporated as above *in vacuo* to dryness, and a known volume (5–10 ml.) of copper phosphate-buffer suspension (Pope & Stevens, 1939) is added to the crystalline residue. Titrations (20–50 μl. 0·1 N-$Na_2S_2O_3$) are carried out on 1 ml. portions after filtering and addition of the other reagents. Neutralization before addition of the copper phosphate is unnecessary.

Procedure for the isolation of cysteic acid. In order to assess the amount of protein from which a workable quantity of cysteic acid may be isolated, its amount in a hydrolysate is roughly estimated by partition chromatography on paper. A known volume (5–10 μl.) is applied to Whatman no. 4 paper and known amounts (0·5–20 μg.) of cysteic acid, each at the same volume as that of the hydrolysate, applied by the side of the hydrolysate spot. After development of the chromatogram in phenol-NH_3-coal gas for 17 hr., the paper is dried, coloured with ninhydrin and the depth of colour of the cysteic acid spot in the hydrolysate compared with those of the controls. The isolation may be conducted either (*a*) by eluting the column with N-HCl after separation of the dicarboxylic amino-acids by method 1 or 2, or (*b*) at pH 1·5. If (*a*) has been used, partition chromatography reveals that the whole of the cysteic acid runs out in the region of the pigment band, at which stage there is a sharp change in pH from 2·5 to <1. No complete separation of the pigment from cysteic acid was achieved by using the resin and eluting solution at a lower pH; however, at pH 1·5 all the amino-acids except cysteic acid are rapidly eluted, thus making possible its isolation on only one column. Thus, on a column, length 30 cm., diameter 0·5 cm., eluted with 0·03 N-HCl, the neutral, basic and dicarboxylic amino-acids accompanied by some pigment, run out in the first 25 ml. followed soon after by cysteic acid, the elution of which can be speeded up by N-HCl.

RESULTS

Recovery of glutamic and aspartic acids from a mixture of amino-acids

An amino-acid mixture simulating a wool hydrolysate (Astbury, 1942) was prepared containing 0·0517 mg. aspartic acid N (4·12 % of total N) and

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* Obtained from the Resinous Products and Chemical Co., Pa.

Table 1. *Recoveries of glutamic and aspartic acids from a synthetic mixture*

		Experiment							
		(i)		(ii)		(iii)		(iv)	
	Amount (mg. N)	Found	Recovery (%)	Found	Recovery (%)	Found	Recovery (%)	Found	Recovery (%)
Glutamic acid	0·8416	0·767	91·3	0·773	92·0	0·776	92·3	0·747	88·8
Aspartic acid	0·4126	0·394	95·2	0·390	94·2	0·404	97·6	0·397	96·0

0·1052 mg. glutamic acid N (8·40 % of total N)/ml. This solution (8 ml.), after evaporation *in vacuo*, was subjected to the foregoing procedure, using method 2. Recoveries of glutamic and aspartic acids are recorded in Table 1.

In experiments on glutamic and aspartic acids alone, similar recoveries were obtained. The odour of formaldehyde could usually be detected in the eluates from the columns after concentration; treatment of the eluates with alkali, HCl, H_2O_2, or dimedone failed to improve the results and treatment with strong NH_3 gave high results. Addition of formaldehyde to glutamic and aspartic acid solutions led to low titrations.

Isolation of cysteic acid

Degraded protein produced by the action of chlorine on wool (Consden *et al.* 1946), containing 156 mg. N and which by the paper chromatographic method described above was expected to contain approx. 4 mg. cysteic acid N, was hydrolyzed and prepared as already described for application to the resin. This material was transferred to a column (30 cm. high; 1·5 cm. diam.) which had been equilibrated to pH 1·5 and was then eluted with 0·03 N-HCl, the rate of flow through the column being about 60 ml./hr. Six eluates of 20 ml. each were collected and tested as described above. The neutral amino-acids plus glutamic and aspartic acids were eluted in the first three fractions. The fourth fraction contained much cysteic acid and subsequent fractions successively less. The column was eluted with a further 500 ml. 0·03 N-HCl and, as small amounts of cysteic acid were still being washed out, it was finally eluted with N-HCl which removed all the remaining cysteic acid and yellow material from the resin. The united fractions containing cysteic acid were concentrated *in vacuo* to small volume and the solution, after standing at 0°, deposited brownish crystals, which after filtration were decolorized by boiling with animal charcoal. The mixture was filtered, evaporated *in vacuo* to dryness and recrystallized three times from aqueous ethanol. The pale yellow powder (15 mg.) had $[\alpha]_D^{18^\circ} + 7{\cdot}1^\circ$ ($l.$ 2; $c = 0{\cdot}3$) in water. Clarke & Inouye (1931) give $[\alpha]_D^{24{\cdot}5^\circ} + 9{\cdot}4^\circ$ for L-cysteic acid. Its equivalent by titration with $Ba(OH)_2$ was 185; calc. for $C_3H_7O_5N.H_2O$, 187; mixed partition chromatograms using phenol-NH_3-coal gas showed no difference from authentic L-cysteic acid. Analysis of the copper salt gave Cu 25·78; calc. for $C_3H_7O_6NSCu$, Cu, 25·57 %.

Titration of Amberlite IR 4

The resin, which had been treated as described at the beginning of the experimental section, was brought to *c.* pH 10 by treatment with dilute NaOH. It was then thoroughly washed with water and left to dry at room temperature. A sample of the resin (202·8 mg.) on drying to constant weight at 105° lost 30·4 mg. Samples of air-dry resin (200 mg.) were mixed with varying quantities of HCl and each made up to a final volume of 20 ml. and allowed to equilibrate overnight. The pH of the filtrate was determined (glass electrode) and acidity by titration against alkali. The absorption of acid by Amberlite IR 4 at different pH values is shown in Fig. 2.

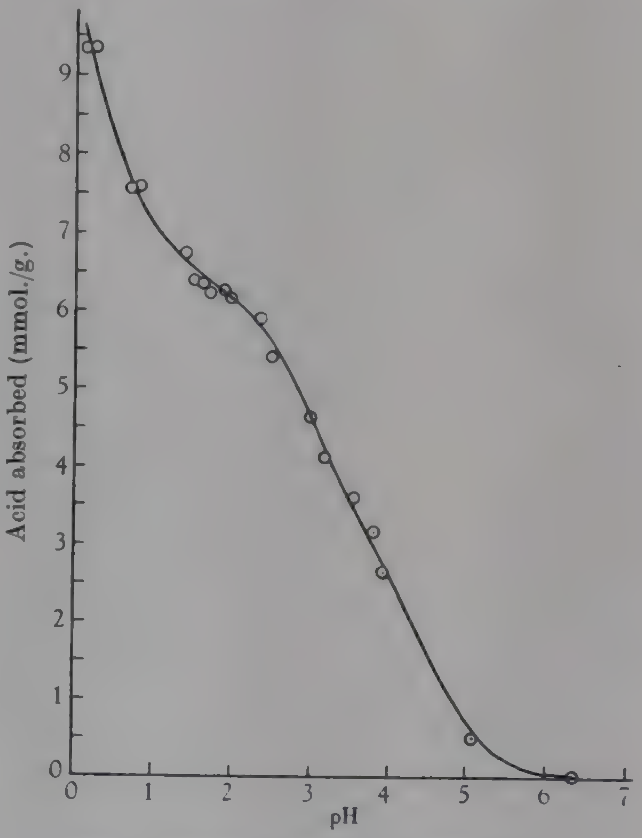

Fig. 2. Titration curve of Amberlite IR 4.

SUMMARY

1. A simple chromatographic method is presented for the separation of glutamic from aspartic acid, and their estimation, after their separation together from a protein hydrolysate, employing the synthetic anion exchange resin, Amberlite IR 4.

2. Optically active cysteic acid was isolated from a hydrolysate of chlorinated wool.

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The Polarographic Estimation of Steroid Hormones

4. DETERMINATION OF 3(α)- AND 3(β)-HYDROXY-17-KETOSTEROIDS

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A knowledge of the urinary excretion of 3(β)-hydroxy-17-ketosteroids ('β-17-ketosteroids') is becoming increasingly important in clinical diagnosis. In addition to the now well-established fact, first suggested by Crooke & Callow (1939) and by Talbot, Butler & Berman (1942), that the relative excretion of β-17-ketosteroids is markedly elevated in patients with adrenal cortical carcinoma, the work of Scott & Vermeulen (1942), Pearlman (1942), Dobriner, Gordon, Rhoads, Lieberman & Fieser (1942), Freeman, Pincus & Glover (1944), Salter, Cahen & Sappington (1946) and Dingemanse, Huis in't Veld & de Laat (1946) has shown that the excretion of β-17-ketosteroids may be altered in various other cancerous and non-cancerous conditions. It therefore appeared desirable to extend the polarographic method for the estimation of 17-ketosteroids (Barnett, Henly & Morris, 1946) so that it could also be used for the routine estimation of the α- and β-17-ketosteroids.

Several methods have been described for the quantitative separation of the 3(α)- and 3(β)-hydroxy fractions of urinary 17-ketosteroid extracts. All depend upon the use of digitonin for precipitation of the β-fraction. The procedure of Talbot, Butler & MacLachlan (1940*a*) is not entirely satisfactory in so far as it relies on an indirect determination of the β-fraction. The micro-method of Baumann & Metzger (1940) has been criticized by Salter *et al.* (1946) on the grounds that several conditions (e.g. ethanolic content of the medium, and initial concentration of steroid) may alter the apparent partition between α- and β-fractions. The procedure of Frame (1944) has the single disadvantage that about 15 mg. of total steroid are required, an amount which would necessitate the extraction of several days' collection of urine from subjects with a low 17-ketosteroid excretion. Pincus (1945) described a microprocedure (using about 1 mg. of total steroid) and suggested the addition of dehydro*iso*androsterone (3(β)-hydroxyandrost-5-en-17-one) in order to obtain maximum precipitation of the β-fraction. He also commented that, regardless of the β-17-ketosteroid content, digitonin precipitation leads to a loss of approximately 0·02 mg. of β-17-ketosteroid to the α-fraction. There is evidently a need for a method of separation of α- and β-17-ketosteroids in urine extracts, which would combine the reliability of

Frame's procedure with the convenience of a micromethod. The method described below (based mainly on the methods of Frame, 1944 and of Pincus, 1945) has been developed with these objects in mind. It has been used in the determination of the excretion of α- and β-17-ketosteroids in a series of ten normal men and ten normal women.

EXPERIMENTAL

Collection and treatment of urine

Ten men (medical students and laboratory staff) and ten women (nurses) provided 24 hr. collections of urine. The specimens were collected in vessels containing 7 ml. of conc. HCl, and the importance of accurate collection was stressed. The specimens were kept in a cool place during collection and then stored in a refrigerator until extracted, generally within 24 hr.

The urines were hydrolyzed and extracted according to the method described by Barnett, Henly & Morris (1946), and the extracts were separated into α- and β-fractions by the procedure described below. The final extracts were oxidized by permanganate-periodate, treated with Girard's reagent *T* and polarographed as described by Barnett, Henly & Morris (1946). The dropping electrode used in the course of this work had the following constants: drop time *t*, 1·68 sec.; average drop weight, 5·35 mg.: capillary constant $k=m^{\frac{2}{3}}\ t^{\frac{1}{6}}=2{\cdot}36$ (m = mass of mercury dropping/sec.). Androsterone and 3(β)-hydroxyandrost-5-en-17-one were found to give polarograms with identical wave heights (100 μg. of each gave a wave height of 17·5 mm. at sensitivity C (cf. Barnett, Henly & Morris, 1946)).

Separation of urinary 17-ketosteroids into α- and β-fractions

Reagents. Digitonin solution. A 1% (w/v) solution of digitonin (British Drug Houses, Ltd.) in warm 90% (v/v) ethanol. The digitonin crystallizes from the solution on standing at room temperature, but it may be kept in solution by storing at 37° in a tightly stoppered container. *Ether*: The ether must be peroxide free. Howard's Standard Ether (*Aether Anaesthethicus P.B.*) has been found satisfactory. *Pyridine*: The 'Analar' reagent is dried over barium oxide and redistilled at frequent intervals. *Sulphuric acid*: Approx. 2 N.

Procedure. A portion of benzene solution of urine extract, containing 1·0–1·5 mg. of 17-ketosteroid, is transferred to a graduated centrifuge tube and evaporated to dryness, preferably under reduced pressure. Warm digitonin solution (0·75 ml.) is added and the mixture quickly heated to boiling (over a microflame) in order to dissolve as much of the material as possible. The tube is stoppered and left in a refrigerator overnight. A total of 10 ml. of peroxide-free ether is then added in small portions, stirring with a thin glass rod between each addition, and allowing the precipitated digitonin and digitonides to flocculate before adding the last 2–3 ml. The mixture is centrifuged for 5 min. at 2000–3000 r.p.m. and the supernatant liquid decanted into a separating funnel of 30–50 ml. capacity. The precipitate in the centrifuge tube is washed three times with 5 ml. portions of ether, stirring and centrifuging each time, as before. The combined supernatant liquids are washed three times with 5 ml. portions of water, transferred to a small flask, the ether removed under reduced pressure, and the residue dried *in vacuo* over $CaCl_2$ for about 1 hr. This constitutes the α-fraction.

The residue in the centrifuge tube is dissolved in 0·25 ml. of dry pyridine and warmed in a water bath at 60–70° for 3 min. After cooling in running water, 5 ml. of ether are added in small quantities, stirring between additions and allowing the precipitate to flocculate as before. The mixture is centrifuged for 5 min. and the supernatant liquid poured off into a small separating funnel. The residue in the centrifuge tube is again treated with pyridine and ether, and the final precipitate washed twice with 5 ml. portions of ether. The combined pyridine-ether extracts are washed twice with 5 ml. portions of $2\,\text{N-}H_2SO_4$ and three times with 5 ml. portions of water. After transferring to a small flask, the ether is removed under reduced pressure and the residue dried *in vacuo* over $CaCl_2$ for about an hour. This constitutes the β-fraction.

With normal urines it will usually be necessary to use about one-tenth of the α-fraction and the whole of the β-fraction for oxidation and polarography. In both cases optimal results are obtained when about 60–120 μg. of 17-ketosteroids are present.

A few experiments have been carried out in which β-fractions obtained from the same urine extract have been polarographed with and without previous oxidation. The results indicate that little improvement may be obtained by oxidation, and further work may show that oxidation of β-fractions is, in fact, unnecessary. It would appear that most of the interfering material removed by oxidation remains with the α-fraction.

RESULTS

Tables 1 and 2 show the results of experiments to test the accuracy of the procedure. In each case the ethanolic solution of pure sterol or urine extract or mixture was evaporated to dryness in a graduated centrifuge tube and separated into α- and β-fractions. Androsterone was used as the α-17-ketosteroid and 3(β)-hydroxyandrost-5-en-17-one as the β-17-ketosteroid. It will be seen that in all cases the recoveries of both fractions are satisfactory.

Table 1. *Recovery of α- and β-17-ketosteroids from pure solutions*

	α-17-Ketosteroids			β-17-Ketosteroids		
		Recovered			Recovered	
Exp. no.	Added (μg.)	(μg.)	(%)	Added (μg.)	(μg.)	(%)
1	—	—	—	100	95	95
2	—	—	—	100	100	100
3	—	0	—	200	200	100
4	100	97	97	100	100	100
5	100	100	100	100	100	100
6	100	97	97	200	194	97
7	60	65	108	100	97	97
8	75	71	95	60	61	102
9	200	200	100	88	80	100
10	400	377	94	50	47	94
	Means		98·7			98·5

Table 2. *Recovery of α- and β-17-ketosteroids from urine extracts*

Exp. no.	α-17-Ketosteroids Present (µg.)	Added (µg.)	Recovery of added material (µg.)	(%)	β-17-Ketosteroids Present (µg.)	Added (µg.)	Recovery of added material (µg.)	(%)
11	914	400	401	100	29	100	91	91
12	914	500	516	103	29	50	50	100
13	914	600	571	95	29	150	141	94
14	1255	150	145	97	36	200	193	97
15	1255	0	0	—	36	500	464	93
16	857	279	283	102	57	300	303	101
17	1614	335	356	106	140	400	414	105
			Means	100·5				97·9

Table 3. *The separation of the 17-ketosteroids in a pooled urine extract into α- and β-fractions, at different concentration levels*

Vol. of extract used (ml.)	Total 17-ketosteroids (µg./ml.)	α-17-Keto-steroids (µg./ml.)	β-17-Keto-steroids (µg./ml.)	(α + β)-17-Keto-steroids (µg./ml.)	(α + β)/total 17-ketosteroids (%)
0·8	1400	1360	82	1442	103·0
1·5	1400	1335	84	1419	101·4
2·5	1400	1300	89	1389	99·2

Table 4. *The urinary excretion of total, α- and β-17-ketosteroids in ten normal men*

Extract no.	Age (yr.)	Volume of urine (ml./24 hr.)	Total 17-ketosteroids (mg./24 hr.)	α-17-Keto-steroids (mg./24 hr.)	β-17-Keto-steroids (mg./24 hr.)	β-17-Keto-steroids (% of total)	Recovery (α + β)/total 17-ketosteroids (%)
MS 1	20	2260	15·1	12·9	0·8	5·3	91
MS 2	20	2270	13·4	10·6	2·0	14·9	94
MS 3	27	1790	21·8	19·3	1·7	7·8	96
MS 4	24	1850	16·9	15·6	0·6	3·6	96
MS 5	31	1920	10·4	9·6	0·8	7·7	100
MS 6	25	950	19·7	18·3	1·2	6·1	99
MS 7	38	1670	11·9	10·4	0·9	7·6	95
MS 8	32	2600	16·3	14·7	0·3	1·8	92
MS 9	31	1310	11·2	9·4	0·7	6·3	90
MS 10	25	1040	15·9	14·7	0·4	2·5	94
Means			15·3	13·6	0·9	6·4	95
Standard deviations (σ)			±3·7	±3·6	±0·5	±3·7	±3
Probable range (mean ±2σ)			7·9–22·7	6·4–20·8	0–1·9	0–13·8	89–101
Standard deviation among duplicates				±0·5	±0·03		

Table 5. *The urinary excretion of total, α- and β-17-ketosteroids in ten normal women*

Exp. no.	Age (yr.)	Volume of urine (ml./24 hr.)	Total 17-ketosteroids (mg./24 hr.)	α-17-Keto-steroids (mg./24 hr.)	β-17-Keto-steroids (mg./24 hr.)	β-17-Keto-steroids (% of total)	Recovery (α + β)/total 17-ketosteroids (%)
WS 1	22	1700	21·4	19·4	1·6	7·5	96
WS 2	20	1200	14·3	13·6	0·6	4·2	99
WS 3	21	1680	10·1	9·4	0·8	7·9	101
WS 4	20	820	8·5	7·7	0·4	4·7	95
WS 5	22	1350	8·5	8·3	0·2	2·4	100
WS 6	18·5	1860	11·9	10·9	0·4	3·4	95
WS 7	21	1250	15·7	15·9	0·4	2·5	104
WS 8	21	775	14·5	13·1	1·0	6·9	97
WS 9	24	710	15·1	14·1	1·0	6·6	100
WS 10	19	2500	20·7	20·2	0·5	2·4	100
Means	20·9	—	14·1	13·3	0·7	4·9	98
Standard deviations (σ)			±4·5	±4·3	±0·4	±2·1	±3
Probable range (mean ±2σ)			5·1–23·1	4·7–21·9	0–1·5	0·7–9·1	92–104
Standard deviation among duplicates				±0·6	±0·03		

Table 6. *The normal urinary excretion of* α- *and* β-*17-ketosteroids as reported by various workers since* 1940

			α-17-Ketosteroids (mg./24 hr.)			β-17-Ketosteroids						Recovery $(\alpha+\beta)$/total 17-ketosteroids		
						(mg./24 hr.)			% of total 17-ketosteroids					
Authors	No. of cases	Sex	Mean	Standard deviation (σ)	Range (mean $\pm 2\sigma$)	Mean	Standard deviation (σ)	Range (mean $\pm 2\sigma$)	Mean	Standard deviation (σ)	Range (mean $\pm 2\sigma$)	Mean (%)	Probable range (%)	Notes
Baumann & Metzger (1940)	12	M.	13·5	3·4	6·7–20·3	0·9	0·9	0–2·7	6·2	5·2	0–16·6	—	—	(1)*
Frame (1944)	2	M.	9·2	—	—	0·4	—	—	2·8	—	—	97	—	(2)
Salter *et al.* (1946)	12	M.	13·0	3·3	6·4–19·6	1·8	1·1	0–4·0	10·7	7·3	0–25·3	94	74–114	(3)
	12	M.	11·9	3·6	4·7–19·1	1·3	0·5	0·3–2·3	9·5	4·2	0·9–17·9	94	78–110	(4)
Butt, Henly & Morris (this paper)	10	M.	13·6	3·6	6·4–20·8	0·9	0·5	0–1·9	6·4	3·7	0–13·8	95	89–101	(5)
Baumann & Metzger (1940)	4	F.	4·4	—	—	0·2	—	—	3·8	—	—	—	—	(6)*
Frame (1944)	4	F.	6·9	—	—	0·14	—	—	1·75	—	—	92	—	(7)
Salter *et al.* (1946)	13	F.	6·5	2·1	2·3–10·7	1·1	0·9	0–2·9	14·4	11·6	0–37·6	96	63–129	(8)
	14	F.	6·5	2·9	0·7–12·3	1·1	0·7	0–2·5	14·9	8·8	0–32·5	96	62–130	(9)
Butt *et al.* (this paper)	10	F.	13·3	4·3	4·7–21·9	0·7	0·4	0–1·5	4·9	2·1	0·7–9·1	98	92–104	(10)

* These authors do not give figures for a separate determination of 'total 17-ketosteroids'. The sum of the α- and β-fractions has been used in the calculation of the percentage β-17-ketosteroids.

(1) Ages of subjects were from 17 to 49 yr. (av. 26·3). Most of the results obtained on 3-day urine collections.
(2) Subjects 52 and 62 yr.
(3) *m*-Dinitrobenzene reaction (Cahen & Salter, 1944)
(4) Antimony trichloride reaction (Pincus, 1943*b*)
(3), (4) On same urine extracts. Subjects aged 20–58 yr. (av. 32·8). Separation into α- and β-fractions carried out by method of Baumann & Metzger (1940).
(5) Polarographic method. Subjects aged 20–38 yr. (av. 27·3).
(6) Subjects aged 26–38 yr. (av. 30). Results obtained on 3-day urine collections.
(7) Subjects aged 23–49 yr. (av. 34·5). Results obtained on 3-day urine collections.
(8) *m*-Dinitrobenzene reaction (Cahen & Salter, 1944)
(9) Antimony trichloride reaction (Pincus, 1943*b*)
(8), (9) On same urine extracts. Subjects aged 22–54 yr. (av. 30·6). Separation into α- and β-fractions carried out by method of Baumann & Metzger (1940).
(10) Polarographic method. Subjects aged 18·5–24 yr. (av. 20·9).

As a further check on the validity of the method, varying quantities of a pooled urine extract were separated into α- and β-fractions by the routine procedure. The results are shown in Table 3. It will be seen that the partition into α- and β-fractions does not appear to be affected by the initial concentration of steroid (cf. the criticism by Salter *et al.* (1946) of the method of Baumann & Metzger (1940)).

The results of the determination of total, α- and β-17-ketosteroids in the urines of ten normal men and ten normal women are shown in Tables 4 and 5. The values quoted for α- and β-17-ketosteroids are the means of duplicate estimations on the same urine extract. The standard deviations among duplicates indicate that reproducibility is satisfactory. It will be seen that the recovery of 17-ketosteroids after fractionation is also satisfactory. In addition it may be noted that the values for total 17-ketosteroids are within the previously published ranges for normal men and women (Barnett, Henly & Morris, 1946; Barnett, Henly, Morris & Warren, 1946).

Although the mean values for the excretion of β-17-ketosteroids are greater for men than for women, both when measured in terms of mg./24 hr. and as percentage of the total ketosteroids, application of Fisher's '*t*' test shows that the differences are not statistically significant.

DISCUSSION

The results of some recent work on the determination of α- and β-17-ketosteroids in normal individuals are collected in Table 6. The data given by the various workers have been used in the calculation of means, standard deviations, etc. In addition the following authors have indicated normal ranges for β-17-ketosteroid excretion without giving individual values: β-fraction usually at least 10–15% of the total 17-ketosteroids (Talbot, Butler & MacLachlan, 1940*b*); β-fraction is 0·6–2·2% of total 17-ketosteroids (Dobriner *et al.* 1942); β-fraction seldom exceeds 15% and is usually 2–3% of the total 17-ketosteroids (Pincus, 1943*a*).

It will be seen from Table 6 that our results for the excretion of α-17-ketosteroids by men are in moderately good agreement with those of other workers. In the case of the women, however, the values found are considerably higher than those found previously. It may be noted that Barnett, Henly, Morris & Warren (1946) found that the polarographic method gave similarly higher results for total 17-ketosteroids, particularly in women.

Our values for β-17-ketosteroid excretion by men show general agreement with those of Baumann & Metzger (1940), but the results obtained by Salter *et al.* (1946) are slightly higher. The corresponding values for women show that the results of Salter *et al.* (1946) are again high, the difference being most marked when the results are expressed as percentage of the total 17-ketosteroids. Some of the normal subjects studied by Salter *et al.* (1946) excreted very large quantities of β-17-ketosteroids. One subject (A.M.) excreted 3·9 mg./24 hr., equivalent to 45·6% of the total 17-ketosteroids, though it may be noted that in this case the sum of the α- and β-17-ketosteroids was some 25% greater than the directly determined value for total 17-ketosteroids (results by *m*-dinitrobenzene reaction).

While each author quoted obtained satisfactory mean recoveries of 17-ketosteroid after fractionation of urine extracts, it is interesting to note the very wide range of recoveries obtained by Salter *et al.* (1946). The reason for this inconsistency is not clear.

Finally, the results described in this paper indicate that the β-17-ketosteroids may contribute up to 15% to the total 17-ketosteroid excretion of normal individuals, though values less than 10% are more usual. No certain sex difference can be demonstrated at the present time. This conclusion is in general agreement with the findings of Baumann & Metzger (1940), Dobriner *et al.* (1942) and Pincus (1943*a*), but is only in partial agreement with the values quoted by Talbot *et al.* (1940*b*).

SUMMARY

1. A micro-method has been described for the quantitative separation of urinary 17-ketosteroid extracts into 3(α)- and 3(β)-hydroxy fractions. The 17-ketosteroids in the separated fractions are estimated polarographically.

2. Recovery of α- and β-17-ketosteroids from pure solutions and from urine extracts is satisfactory.

3. The urinary excretion of α- and β-17-ketosteroids has been determined in a series of ten normal men and ten normal women, and the results have been compared with those obtained by other workers.

4. It is concluded that the total 17-ketosteroids excreted by normal individuals may contain up to 15% of 3(β)-hydroxy compounds, though values less than 10% are more general. There is no significant difference between the β-17-ketosteroid excretion of men and women.

The authors wish to express their thanks to the men and women who co-operated in the collection of 24 hr. urine specimens.

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Studies in Human Mineral Metabolism

1. THE EFFECT OF BREAD RICH IN PHYTATE PHOSPHORUS ON THE METABOLISM OF CERTAIN MINERAL SALTS WITH SPECIAL REFERENCE TO CALCIUM

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The discovery that phytic acid forms relatively insoluble salts with several of the common bases, that are of importance in our diet, has led to several investigations in which the availability of these bases has been determined with varying intakes of phytic acid. The conclusions drawn from such work have been somewhat contradictory: thus, whilst all observers are agreed that the addition of a considerable amount of phytate P is invariably followed by an immediate reduction in the amount of Ca retained, some investigators have suspected, and others are emphatic, that, given time, an adaptation to such a change can occur, and that eventually the body is able to adjust itself fairly satisfactorily to the new conditions.

Obviously laboratory investigations must eventually be reconciled with what happens in everyday life, and a consideration of those human diets which are rich in cereals and hence in phytic acid, yet poor in Ca, lends a considerable amount of support to the view that some such process of adaptation must in fact take place. Otherwise it is difficult to explain how the consumers avoid the disastrous consequences which would arise if part or the whole of this Ca were rendered unavailable.

For two reasons this issue is of particular importance to a country like South Africa. In the first place the large majority of the population, including most of the non-Europeans and not a few of the poorer Europeans, live on just such a diet as has been mentioned above. Secondly, in 1941, a compulsory Standard War Bread, made from 95 to 100% extraction meal, was introduced and is still the only kind of bread allowed to be baked.

As a result of the work published by McCance & Widdowson (1942), the South African National Nutrition Council decided that it was imperative to study the problem under local conditions. For this purpose balance experiments, similar to those described by these workers, were initiated to observe the effects of breads made from high and low extraction meals upon mineral metabolism in adult male subjects.

EXPERIMENTAL

Plan of the experiments. The intake and excretion of Ca, Mg, total P, phytate P and Fe, were determined on four healthy adult European males, over periods ranging from 3 to 19 *consecutive* weeks.

The experimental periods were planned as follows: *A*, from 1 to 2 weeks on the usual everyday diet; *B*, from 4 to 9 weeks on a diet which contained 1 lb. of Standard War Bread (experimental diet no. 1); *C*, from 1 to 4 weeks in which the war bread was replaced by 1 lb. of white bread (70% extraction; experimental diet no. 2). For the first subject only the first two of these periods were observed, as this part of the experiment was of a preliminary nature carried out to gain acquaintance with the procedure.

The balance procedure and the analytical methods employed followed those described by McCance & Widdowson (1942), except in the following details: (i) Tap water was used throughout. (ii) Experience showed that the analytical procedure was facilitated by keeping the food samples separate for the following groups: milk, drinks, fatty foods, eggs, bread, and the remaining foodstuffs, i.e. soup, meat, vegetables, fruit, puddings and so forth. To obtain a thoroughly representative sample this last group was taken to dryness and finely ground. (iii) The total faeces for the week were also taken to dryness and finely ground before sampling.

Subjects. Three of the subjects were research biochemists and the fourth a reliable laboratory technician. During the investigation they lived in their homes and pursued their usual avocations. Health remained excellent in all cases throughout the investigation; no digestive or other disturbances were reported, and fluctuations in weight were insignificant. Some inconvenience was experienced in consuming the required amount of bread, but apart from the tedium inevitably associated with the daily weighing of all foods, etc., no difficulties were encountered.

RESULTS

Phytate-phosphorus content of meal and bread

Analysis of 12 samples of our meal showed that it contained an average of 205 mg. phytate P/100 g. as compared with 214 mg. found for the overseas 92% extraction product. In addition, when samples of bread obtained from bakeries throughout the country were analyzed, it was found that the average figure for 33 loaves was 50 mg. phytate P for 100 g. of fresh bread, as against 'about 100 mg.' found by McCance & Widdowson. Hence our meals were not only no richer in phytate P, but evidently a far greater destruction of this substance had occurred during the baking than in the case of the British 92% extraction loaf.

For the sake of brevity the terms 'brown' and 'white' breads are employed when referring to the 95–100% and the 70% extraction breads respectively.

Results of balance experiments

In view of the recent findings of Steggerda & Mitchell (1946*b*) that faecal Ca may not represent only unabsorbed Ca, attention has been focused solely upon the daily balance of the various elements.

Calcium metabolism

The results are given in Table 1.

A. *Period on the usual diet.* This period was included in order to evaluate the subject's nutritional state before the experimental diets were given. It will be seen that the subjects A.W., L.O. and L.G. were all in slight positive balance.

B. *Experimental diet no.* 1. The subjects were required to consume 1 lb. of Standard War Bread daily, but otherwise they were permitted to eat what they liked, except that the total Ca intake was regulated by adjusting the amount of milk and cheese consumed so as to comply with the requirements based on body weight (10 mg./kg. daily; see p. 458).

Subject F.F. As mentioned above, this study was of a preliminary nature. The subject immediately went into negative Ca balance, which however became less as the experiment proceeded. It was this fact, together with the gradual improvement in the amount of Ca retained, which made us realize the necessity for continuing our observations for prolonged periods. Accordingly it was decided to commence a more lengthy study, during which the degree of adjustment to each change in diet could be followed as quickly as analytical determinations of Ca would permit. In practice it was found possible to be not more than 1 week behind with these determinations. This enabled us to decide when a steady state had been reached, so that the next part of the investigation could be safely proceeded with.

Subject A.W. For no less than 4 weeks this subject remained in continuous negative balance (designated period B_1); this was followed by a further 5 weeks (designated period B_2) during which the balance was continuously positive, or almost positive. If McCance & Widdowson's (1942) suggested limits of significance of ± 25 mg./day are accepted, this subject may be regarded as having reached a state of equilibrium.

Subject L.O. This subject needed 5 weeks to adjust to the new diet (period B_1) and he then remained in equilibrium for the following 3 weeks (period B_2). It must, however, be mentioned that he experienced some difficulty in consuming the full 1 lb. of war bread daily, and hence his intake of other foods tended to suffer. In order to supply the daily requirement of Ca estimated on body weight, a small additional allowance of milk was therefore permitted after the 5th week.

Subject L.G. This subject proved of particular interest to us, since his Ca intake on his usual diet was found to be only just adequate for maintenance. With the experimental diet his Ca intake remained unchanged, and hence, unlike the other subjects, he only had to adjust to the increased ingestion of phytate P. It is presumably for this reason that he

Table 1. *Daily intake, excretion, and retention of calcium and magnesium, from the usual, brown-bread, and white-bread diets*

Period			(1) Calcium					(2) Magnesium				
Duration and sequence	Designation	Diet consumed	Intake (mg.)	Urine (mg.)	Faeces (mg.)	Total excretion (mg.)	Balance (mg.)	Intake (mg.)	Urine (mg.)	Faeces (mg.)	Total excretion (mg.)	Balance (mg.)
(days)		Subject F.F. Weight 65 kg. Age 50 years										
3	*A*	Usual diet	—	225	914	1139	—	—	98	300	398	—
4	*B*	Experimental diet no. 1 including 1 lb. of standard war bread daily	535	192	504	696	-161	490	108	372	480	+ 10
7	*B*	,, ,, ,,	501	235	512	747	-246	480	89	395	484	- 4
7	*B*	,, ,, ,,	490	210	350	560	- 70	535	91	440	531	+ 4
4	*B*	,, ,, ,,	533	205	390	595	- 62	560	93	450	543	+ 17
		Average figures for period *B*	510	214	437	651	-141	514	94	415	509	+ 5
(weeks)		Subject A.W. Weight 51 kg. Age 31 years										
1	*A*	Usual diet	1160	246	848	1094	+ 66	495	80	405	485	+ 10
2	*A*	,,	1175	256	865	1121	+ 54	490	81	415	496	- 6
		Average figures for period *A*	1167	251	856	1107	+ 60	492	80	410	490	+ 2
3	B_1	Experimental diet no. 1 including 1 lb. of standard war bread daily	510	194	402	596	- 86	525	86	450	536	- 11
4	B_1	,, ,, ,,	512	203	387	590	- 78	550	79	620	699	-149
5	B_1	,, ,, ,,	524	220	373	593	- 69	575	126	570	696	-121
6	B_1	,, ,, ,,	501	269	297	566	- 65	575	133	545	678	-103
		Average figures for period B_1	512	221	365	586	- 74	556	106	546	652	- 96
7	B_2	Same as in period B_1	509	225	255	480	+ 29	500	59	430	489	+ 11
8	B_2	,, ,,	515	217	320	537	- 22	585	75	560	635	- 50
9	B_2	,, ,,	509	201	321	522	- 13	575	79	485	564	+ 11
10	B_2	,, ,,	561	219	295	514	+ 47	580	87	475	562	+ 18
11	B_2	,, ,,	488	196	257	453	+ 35	500	77	415	492	+ 8
		Average figures for period B_2	516	212	289	501	+ 15	547	75	473	548	- 1
12	C_1	Experimental diet no. 2*a* including milk, white rolls and potatoes daily	440	170	235	405	+ 35	310	76	225	301	+ 9
13	C_1	,, ,, ,,	486	145	272	417	+ 69	337	91	235	326	+ 11
14	C_1	,, ,, ,,	491	183	285	468	+ 23	300	75	210	285	+ 15
15	C_1	,, ,, ,,	499	170	260	430	+ 69	275	80	185	265	+ 10
		Average figures for period C_1	479	167	263	430	+ 49	305	80	214	294	+ 11
16	C_2	Experimental diet no. 2*b* including 1 lb. of white bread daily	512	245	235	480	+ 32	290	75	195	270	+ 20
17	C_2	,, ,, ,,	503	260	230	490	+ 13	285	80	195	275	+ 10
18	C_2	,, ,, ,,	471	200	225	425	+ 46	250	56	190	246	+ 4
19	C_2	,, ,, ,,	498	155	255	410	+ 88	245	36	195	231	+ 14
		Average figures for period C_2	496	215	236	451	+ 45	268	62	194	256	+ 12

		Subject L.O. Weight 64 kg. Age 28 years										
1	*A*	Usual diet	1090	139	936	1075	+ 15	512	146	360	506	+ 6
2	*A*	,,	927	132	780	912	+ 15	435	92	335	427	+ 8
		Average figures for period *A*	1008	135	858	993	+ 15	473	119	347	466	+ 7
3	B_1	Experimental diet no. 1 including 1 lb. of standard war bread daily	465	117	355	472	− 7	410	145	295	440	− 30
4	B_1	,, ,, ,,	485	123	375	498	− 13	440	190	322	512	− 72
5	B_1	,, ,, ,,	506	149	394	543	− 37	480	138	380	518	− 38
6	B_1	,, ,, ,,	504	155	367	522	− 18	508	130	430	560	− 52
7	B_1	,, ,, ,,	465	141	450	591	− 126	550	101	510	611	− 61
		Average figures for period B_1	485	137	388	525	− 40	478	141	387	528	− 50
8	B_2	Same as in period B_1	620	133	480	613	+ 7	551	110	440	550	+ 1
9	B_2	,, ,,	630	97	520	617	+ 13	482	70	425	495	− 13
10	B_2	,, ,,	660	140	510	650	+ 10	566	120	428	548	+ 18
		Average figures for period B_2	636	123	503	626	+ 10	533	100	431	531	+ 2
11	*C*	Experimental diet no. 2 including 1 lb. of white bread daily	750	182	517	699	+ 51	318	111	202	313	+ 5
12	*C*	,, ,, ,,	720	170	535	705	+ 15	278	115	166	281	− 3
13	*C*	,, ,, ,,	695	110	495	605	+ 90	265	102	153	255	+ 10
		Average figures for period *C*	722	154	516	670	+ 52	287	109	174	283	+ 4
		Subject L.G. Weight 75 kg. Age 35 years										
1	*A*	Usual diet	763	193	537	730	+ 33	443	134	335	469	− 26
2	*B*	Experimental diet no. 1 including 1 lb. of standard war bread daily	740	269	550	819	− 79	790	137	584	721	+ 69
3	*B*	,, ,, ,,	719	220	504	724	− 5	677	172	528	700	− 23
4	*B*	,, ,, ,,	705	210	485	695	+ 10	735	175	518	693	+ 42
5	*B*	,, ,, ,,	880	233	595	828	+ 52	755	190	560	750	+ 5
		Average figures for period *B*	761	233	533	766	− 5	739	169	547	716	+ 23
6	*C*	Experimental diet no. 2 including 1 lb. of white bread daily	890	320	535	855	+ 35	465	185	270	455	+ 10

required only 3 weeks to reach equilibrium. After 2 weeks of positive Ca balance it was considered permissible to proceed with the next experimental diet.

C. *Experimental diet no. 2. Subject A.W.* Before passing on to the white-bread diet, this subject for a period of 4 weeks was given a diet in which the Ca supplied by the war bread was replaced mainly from milk. The supply of calories was maintained by means of white rolls and extra potatoes (period C_1). The amount of phytate P was thus very considerably reduced, although not as much as might be expected, since white rolls contain more of this substance than white bread made from the same flour. The Ca retention of the first balance period on this diet was the same as that of the last of the previous series. Thereafter the balances became more positive.

After this period of 4 weeks, the subject was placed on a diet in which the rolls and extra potatoes were replaced by 1 lb. white bread (period C_2). It will be seen that the retention of Ca was hardly affected. This period was continued for 4 weeks, and the consistency of the results showed that the subject was well adjusted to the change.

Subjects L.O. and L.G. changed over directly to the white-bread diet without the intervening period C_1. (It is unfortunate that, for reasons beyond our control, both L.O. and L.G. inadvertently consumed slightly more Ca than had been intended.) L.O. showed a slight improvement in Ca retention, while L.G. was virtually in equilibrium in the single balance period in which he was studied.

Magnesium metabolism

The intake of Mg, as will be seen from Table 1, even on the white-bread diet, was always ample for maintenance, i.e. it exceeded 250 mg. daily, the standard suggested by Duckworth & Warnock (1942).

McCance & Widdowson's (1942) subjects were in equilibrium throughout their experiments, but we found, on passing from the usual to the brown-bread diet, that two of our subjects began to lose more Mg than they ingested. As with Ca, however, Mg retention gradually improved. The urinary excretion of Mg was markedly increased during part of the brown-bread-diet period. During the white-bread period, the same total amount was retained as in the previous period, although the intake was only half of that on the brown-bread diet. The possible effect of variations in the Mg intake upon the utilization of Ca is referred to in a later section.

Total phosphorus metabolism

For the sake of brevity the metabolism data for total P and phytate P have been given in Tables 2 and 3 as averages for the different dietetic periods. This course was adopted because the weekly figures during those periods differed little from one another.

The metabolism of total P showed least disturbance of all the elements studied with the changes in diet. Although the amount consumed on the white-bread diet was less than that on the brown-bread diet, the retention rose somewhat. McCance & Widdowson (1942) found the same balance with either type of bread.

Table 2. *The daily intake, excretion, and retention of total phosphorus from the usual, brown-bread, and white-bread diets*

Period							
Duration	Designation	Diet consumed*	Intake (mg.)	Urine (mg.)	Faeces (mg.)	Total excretion (mg.)	Balance (mg.)
(days)	Subject F.F.*						
3	*A*	Usual diet	—	1520	650	2170	—
22	*B*	Brown-bread diet	885	1435	454	1889	− 4
(weeks)	Subject A.W.*						
2	*A*	Usual diet	1620	1020	542	1562	+ 58
4	B_1	Brown-bread diet	1415	870	529	1399	+ 16
5	B_2	Brown-bread diet	1380	862	483	1345	+ 35
4	C_1	Diet of milk, white rolls and potatoes	1107	760	291	1051	+ 56
4	C_2	White-bread diet	995	685	239	924	+ 71
	Subject L.O.*						
2	*A*	Usual diet	1495	740	752	1492	+ 3
5	B_1	Brown-bread diet	1559	1022	579	1601	− 42
3	B_2	Brown-bread diet	1716	1008	663	1671	+ 45
3	*C*	White-bread diet	1178	705	395	1100	+ 78
	Subject L.G.*						
1	*A*	Usual diet	1600	1000	560	1560	+ 40
4	*B*	Brown-bread diet	1825	1097	769	1866	− 41
1	*C*	White-bread diet	1540	930	500	1430	+110

* For fuller details see Table 1.

Table 3. *The daily intake, excretion, and retention of phytate phosphorus from usual, brown-bread, and white-bread diets*

Period					
Duration	Designation	Diet consumed*	Intake (mg.)	Faeces (mg.)	Phytate hydrolyzed (%)
(days)	Subject F.F.*				
3	*A*	Usual diet	—	135	—
22	*B*	Brown-bread diet	425	56	87
(weeks)	Subject A.W.*				
2	*A*	Usual diet	178	65	64
4	B_1	Brown-bread diet	289	33	88
5	B_2	Brown-bread diet	280	24	91
4	C_1	Diet of milk, white rolls and potatoes	84	26	69
4	C_2	White-bread diet	70	6	91
	Subject L.O.*				
2	*A*	Usual diet	142	53	63
5	B_1	Brown-bread diet	289	26	90
3	B_2	Brown-bread diet	345	38	89
3	*C*	White-bread diet	41	22	46
	Subject L.G.*				
1	*A*	Usual diet	105	60	43
4	*B*	Brown-bread diet	315	63	80
1	*C*	White-bread diet	90	50	44

* For fuller details see Table 1.

Table 4. *The daily intake, excretion, and retention of iron, from usual, brown-bread, and white-bread diets*

Period							
Duration	Designation	Diet consumed*	Intake (mg.)	Urine (mg.)	Faeces (mg.)	Total excretion (mg.)	Balance (mg.)
(days)	Subject F.F.*						
3	*A*	Usual diet	—	1·0	20·5	21·5	—
22	*B*	Brown-bread diet	26·1	0·4	24·6	25·0	+1·1
(weeks)	Subject A.W.*						
2	*A*	Usual diet	20·5	0·5	19·7	20·2	+0·3
4	B_1	Brown-bread diet	26·0	0·4	25·0	25·4	+0·6
5	B_2	Brown-bread diet	23·9	0·7	22·7	23·4	+0·5
4	C_1	Diet of milk, white rolls and potatoes	14·4	0·2	13·5	13·7	+0·7
4	C_2	White-bread diet	16·0	0·2	15·0	15·2	+0·8
	Subject L.O.*						
2	*A*	Usual diet	14·3	1·1	13·0	14·1	+0·2
5	B_1	Brown-bread diet	19·2	1·3	17·9	19·2	±0·0
3	B_2	Brown-bread diet	23·1	0·9	21·8	22·7	+0·4
3	*C*	White-bread diet	15·1	1·3	13·1	14·4	+0·7
	Subject L.G.*						
1	*A*	Usual diet	18·3	1·2	16·2	17·4	+0·9
4	*B*	Brown-bread diet	25·8	1·4	24·1	25·5	+0·3
1	*C*	White-bread diet	22·7	2·7	19·0	21·7	+1·0

* For fuller details see Table 1.

Phytate-phosphorus metabolism

When the Ca intake was high and that of phytate P comparatively low (i.e. on the usual diet), an average of 59 % of the phytate P was hydrolyzed, whereas when the Ca intake was just adequate for maintenance and phytate-P intake high, an average of 84 % was hydrolyzed. Judging by the conclusions reached by Harrison & Mellanby (1939), one might argue that in the absence of an abundance of Ca, the phytate P forms more readily hydrolyzable salts with bases such as Na and K. The condensed arrangement of the tables, however, masks the interesting fact that, after the change from the usual to the brown-bread diet, the amount of phytate P hydrolyzed reached its maximum extent *immediately*, without the incremental steps of adjustment found for Ca and Mg.

Iron metabolism

Following the suggestion made by McCance & Widdowson (1942), we carried out Fe balances for each subject, mainly because they afforded a simple means of checking the accuracy of the experimental procedure, more especially the marking off of the different faecal periods. The results (Table 4) show that the consumption of brown bread did not affect the amount of Fe retained. Such a conclusion would tend to support the work of several investigators who have found that the consumption of higher extraction breads had no deleterious effect upon the retention of this metal.

DISCUSSION

Environment

When a comparison is made between the experiments of McCance & Widdowson (1942) and our own, it should be pointed out that the present study extended over the different seasons. Owing to our climatic conditions, the subjects not only consumed food which had been fully irradiated by the sun, but were themselves continuously exposed to bright light at an altitude of approximately 6000 feet. There was thus this difference between the two studies, but, as shown by the very full review given by McCance & Widdowson, there is little evidence to suggest that the addition of vitamin D to the diet of healthy adults has any appreciable effect upon the absorption of Ca.

Calcium balances

At the outset we desire to draw attention to certain sources of error inherent in balance studies of this particular kind, which do not always appear to be sufficiently appreciated.

In recent years evidence has been accumulating that, when an individual who is habituated to one level of Ca intake is suddenly placed on a lower level, a period of adjustment is inevitable and may even be somewhat prolonged (Nicholls & Nimalasuriya, 1939; Cathcart, 1940; Steggerda & Mitchell, 1941; Kraut & Wecker, 1943). According to these workers the time taken to reach a steady state at the new level of Ca intake depends upon the magnitude of the difference between the two levels. It must also depend upon the extent to which the new supplies of Ca are available. Thus, if the reduction of the Ca intake is accompanied by an increase in the phytate-P intake, the negative balances following such changes cannot be interpreted as being solely due to the interfering effects of the phytate P.

Also, when passing from a high to a lower Ca intake it is necessary to insure that at least enough Ca is provided to satisfy the minimum requirements of the individual concerned. Otherwise the negative balances that will be observed are likely to be due to what may be termed an 'excretion momentum', in addition to any effects arising from a change in the availability of the supply. The difficult question of what constitutes a minimum daily requirement of Ca has recently been the subject of intensive study by Leitch (1937), Holmes (1945) and more especially Mitchell and his associates (Mitchell & Curzon, 1939; Steggerda & Mitchell, 1939, 1941, 1946*a*). These workers have reached substantial agreement with a figure of about 10 mg./kg. body weight, and we accordingly adopted this standard when planning our experiments.

Furthermore, in the reports given by some investigators the experimental periods are interrupted by days or weeks during which the food consumed is not specified. Unless the same level of Ca intake is maintained it seems inevitable that such interruptions will affect the subsequent observations. Lastly, there are other workers who employ such short experimental periods, and change the diets so frequently, that it is questionable whether the body has time to adjust itself to one diet before the next is taken. An extreme example of this is to be seen in the study reported by Wang, Liu, Chu, Yu, Chao & Hsu (1944), where, within the space of 38 days the diet was changed no less than twelve times.

The assimilation of calcium in the presence of phytic acid

Although our main experiments were carried out on only three subjects, the results obtained confirmed one another.

It is not easy to compare our findings with those reported by other observers because of the comparatively short experimental periods usually employed by them. Thus, whilst the experiments reported by McCance & Widdowson (1942) lasted intermittently over 9 months, the actual periods of continuous observation were usually 3–4 weeks. These workers were emphatic that there was no indication of any adaptation occurring. Similarly, the experimental periods employed by Krebs & Mellanby (1943) lasted for 4 weeks; but it is worth noting that before the experiments on the 85% extraction bread terminated, one of their six volunteers (no. 3) was already showing improvement in the retention of Ca and finally reached equilibrium. In their recent experiments with children, Hoff-Jørgensen, Andersen & Nielsen (1946) suspected that the retention of Ca was gradually improving with time. (We presume, although this is not stated, that the same diet, rich in phytic acid, was continued during the short intervals that occurred between their 5-day periods.) Indeed, had we discontinued our observations after 3–4 weeks on the high-extraction bread diet, our findings would have been very similar to those obtained by these three groups of investigators.

Combining our own evidence with that already available, we suggest the following: subjects passing abruptly from their usual diet to one containing less Ca and much more phytate P show an immediate negative Ca balance. As the body becomes accustomed to such a diet, the retention of Ca improves, so that, *given time*, equilibrium again occurs and the losses of Ca are slowly made good. The period of adjustment is shorter, and the loss of Ca is less, if the disparity between the usual Ca intake and that of the experimental diet is small.

Evidence available from studies made on individuals habituated to diets high in phytate phosphorus and low in calcium.

There is, however, another method of approach which throws valuable light on the whole problem. Instead of placing a few selected individuals on prescribed diets and then observing how they adjust themselves to the new conditions over shorter or longer periods (which for obvious practical reasons can never be really very prolonged relative to the life span), it ought to be possible to gain more reliable information by studying people who, through force of circumstances, are compelled to subsist for long periods on diets which are very rich in phytate P and very low in Ca. Some of the few available Ca balance studies that are on record for such people have been assembled in Table 5.

Although we have deliberately selected examples where the Ca balances were positive, it may be stated that this obtained in all the cases reported by Nicholls & Nimalasuriya (1939); in two out of the five cases reported by Henderson & Kelly (1929–30) for boys on prison diet; in two out of four adult cases on the same type of diet (Kelly & Henderson, 1929–30); and in two out of the three subjects studied by Basu, Basak, & Sircar (1939). This must presumably indicate that such positive balances occur fairly frequently. Even the survival of these individuals on such diets is remarkable. That they are somewhat shorter and lighter than well nourished Europeans is admitted, but with a diet lacking in so many other respects it is hardly fair to attribute this solely to a lack of available Ca. As far as our rural South African Bantu are concerned there is not much evidence of poor skeletal calcification, whilst the teeth are often well formed and sometimes remarkably free from caries. Thus in a recent careful dental survey carried out on some 1000 Bechuanaland children, whose diet consists mainly of Kaffir corn, no less than 70% were found to be free from all signs of caries. Equally striking results have been reported by Wilson & Widdowson (1942) for Indian children.

It must be admitted that such observations cannot easily be reconciled with orthodox opinions; however, we do not consider it satisfactory to dispose of them as being due to racial differences, but think rather that they reflect an adaptation which any human being may be capable of making under similar circumstances. That somewhat similar adaptation can be achieved by Europeans is suggested by the experience of the French people during the occupation. They consumed a diet extremely low in Ca and including high-extraction bread (Paris

Table 5. *Examples of positive calcium balances observed with non-Europeans of different races when consuming their typical cereal diets, rich in phytate phosphorus and low in calcium*

Diet and authority	Sex and age (yr.)	Experimental period (days)	Intake (mg./day)	Urine (mg./day)	Faeces (mg./day)	Total excretion (mg./day)	Balance (mg./day)
(1) Nicholls & Nimalasuriya (1939). Sinhgalese children consuming usual diet of cereals, legumes, roots and vegetables	*M* 4	3	205	25	55	80	+125
	M 4	3	183	24	96	120	+ 63
	F 7	3	245	29	135	164	+ 81
	M 7	3	70	4	15	19	+ 51
	M 7	3	223	7	47	54	+169
	M 7	3	220	5	45	50	+170
(2) Henderson & Kelly (1929–30). Bantu boys consuming long term prison diet including 1·5 lb. maize, beans, potatoes, fat and meat. Average of two 4-day periods taken during a 21-day study	*M* 16	8	300	23	271	294	+ 6
	M 16	8	300	19	242	261	+ 39
(3) Kelly & Henderson (1929–30). Bantu adults consuming diet almost the same as in (2). Average of a 4-day period taken once during a 42-day study. Prison diets (2) and (3) both resembled diets commonly consumed	*M*	4	300	23	151	174	+126
	M	4	300	10	172	182	+118
(4) Basu *et al.* (1939). Indian adults. Diet (*a*) included 400 g. rice; average of 6-day period. Diet (*b*) included 600 g. whole wheat: average of 12 consecutive days	(*a*) *M*	6	280	70	162	232	+ 48
	(*b*) *M*	12	310	19	167	186	+124

letter, 1942), but it is claimed that the decalcification which might have been expected did not take place (Paris letter, 1946).

It is possible that some similar process of adaptation had occurred, though to a lesser extent, in our subjects, since for several years the inhabitants of South Africa have been restricted solely to the use of high extraction bread, and hence some degree of habituation to it may have occurred. This capacity for adjustment may well have been lacking in the subjects studied by McCance & Widdowson (1942).

The mechanism of adaptation to diets with a high phytic acid content

This adaptation can be explained in two ways: one based on our knowledge of the chemical changes which phytates may undergo in the intestine, the other on the body's known ability to adapt itself to a lower intake of Ca.

(*a*) *The digestion of phytate phosphorus.* Most workers seem to agree that phytate P and Ca combine to form an insoluble salt in the intestine. Thus Harrison & Mellanby (1939) from their work on puppies remark: 'It is not possible to say whether such actual precipitation occurs under the conditions present in the gut, but it seems not unlikely.' McCance (1946), discussing the position as far as humans are concerned, concludes that phytic acid 'precipitates Ca in the intestine and by so doing prevents its absorption'. Assuming that the pH values in the intestinal tract of man resemble those observed in the pig, Møllgaard, Lorenzen, Hansen & Christensen (1946) conclude that 'phytic acid may precipitate Ca even in the first part of the small intestine thus causing a serious fall in its absorption'. Hence the consensus of opinion is definitely that the precipitation of Ca by phytic acid occurs, and the fact must therefore be accounted for that much of this Ca is eventually absorbed.

There is general agreement that some degree of hydrolysis of phytate P occurs in the digestive tract. This has been demonstrated for pigs, rats, dogs and hens. Similar results for man have been reported by McCance & Widdowson (1935), Wang *et al.* (1944), Cruickshank, Duckworth, Kosterlitz & Warnock (1945), and recently for babies and children by Hoff-Jørgensen, Andersen, Begtrup & Nielsen (1946) and Hoff-Jørgensen, Andersen & Nielsen (1946).

As far as animals are concerned, the extent to which hydrolysis occurs appears to depend on the level of the Ca intake, e.g. for rats, hens and dogs it has been found that the lower the Ca intake the greater the amount of phytate P hydrolyzed. Likewise for man Cruickshank *et al.* (1945), in a study in which the cereal employed was oatmeal, found that the amount of phytate P hydrolyzed was inversely proportional to the Ca intake. Similar results are reported in the present paper.

At present there are two views as to how this hydrolysis is brought about in man. Some maintain that it is due to the action of the intestinal flora in the digestive tract, whilst Møllgaard *et al.* (1946) insist that hydrolysis can only occur when the diet contains a specific enzyme, which they regard as being normally destroyed when food is cooked.

Both in the case of animals and man there seems to be no difference of opinion that at least a proportion of the P liberated by hydrolysis is absorbed. The question at issue is whether hydrolysis occurs at such a level as will also permit of the liberated Ca being absorbed.

It has been shown that under certain circumstances this does take place in the case of pigs, rats, hens and dogs, and it is of interest to note that Hoff-Jørgensen, Andersen, Begtrup & Nielsen (1946) conclude from their experiments on children 'that a small part of the Ca which was precipitated as Ca phytate was absorbed'. Similarly, Wilson & Widdowson (1942) reviewing their Indian observations, where they were dealing with diets which contained a great deal more phytic acid P than was sufficient to precipitate all the Ca present, came to the same conclusion.

A further interesting point arises from a consideration of the part Mg may play in this connexion. It is more than likely that the phytate P precipitates in the intestinal tract a compound which is not solely the Ca salt, as seems to be assumed by Hoff-Jørgensen (1946), Hoff-Jørgensen, Andersen, Begtrup & Nielsen (1946) and Hoff-Jørgensen, Andersen & Nielsen (1946). Both Harrison & Mellanby (1939) and McCance & Widdowson (1942) have shown that when precipitation occurs *in vitro* in the presence of both bases, a mixed phytate is formed containing approximately equal equivalents of Ca and Mg. The latter workers, who attempted to simulate the conditions which occur in the intestinal tract in regard to pH value, are of the opinion that a precipitate of this nature is likely to be formed. Obviously this is an important point, for it would decide the amount of Ca made less available by a given amount of phytate P. Our own results tend to support the proposition that phytic acid reacted with Mg as well as with Ca.

(*b*) *The ability of the body to adapt itself to a lowered calcium intake.* Irrespective of the composition of the diet, it is now well known that the body can adapt itself to a Ca intake often well below 10 mg./kg. body weight daily. This has been stressed by Nicholls & Nimalasuriya (1939), Owen, Irving & Lyall (1940), Steggerda & Mitchell (1941, 1946*a*) and Kraut & Wecker (1943). This adaptation, in the present experiments, was not due only to increased hydrolysis of phytate, since the increased hydrolysis took place immediately the brown-bread regime was instituted, whereas the adaptation was gradual.

The ability of the body in this respect has been well summarized by Steggerda & Mitchell (1941) as follows: 'It appears that in the presence of an inadequate supply of any nutrient, including Ca, the body can adjust itself to the situation, either by a more economical use of what little is available, or by a lowering of its own requirements, so that eventually it comes into equilibrium with the limited food supply.' These writers (Steggerda & Mitchell, 1946*a*) consider that no harm can come from such an adaptation, provided the Ca intake is 10 mg./kg. body weight daily.

This work was originally undertaken to find out if the results of McCance & Widdowson (1942) applied in South Africa. It is evident that our results confirm those of these workers in so far as we have shown that a high-brown-bread regime renders a certain amount of the dietary Ca less available. However, further evidence shows beyond doubt that the body can adjust to this lessened availability, and that the consumption of such a diet over long periods has no deleterious effect upon Ca metabolism.

SUMMARY

1. The metabolism of calcium, magnesium, phosphorus, phytate phosphorus, and iron has been followed in three healthy adult European males for periods lasting from 7 to 19 *consecutive* weeks. During this time the subjects consumed (*a*) their usual diet, (*b*) a diet including 1 lb. of bread made from 95 to 100% extraction meal, and (*c*) a diet including 1 lb. of bread made from 70% extraction meal. During dietary periods (*b*) and (*c*) the daily calcium intake was restricted to a level of approximately 10 mg./kg. of the subject's body weight.

2. When the subjects passed abruptly from their usual diet to one containing much more phytate phosphorus and, in two cases, less calcium, they showed an immediate negative calcium balance.

3. As they became accustomed to such a change, the retention of calcium improved, so that, *given time*, equilibrium was again reached and the losses of calcium were slowly made good.

4. The period of adjustment was shorter, and the loss of calcium was less, if the disparity between the usual calcium intake and that from the experimental diet was small.

5. The lower the calcium content of the diet, the greater was the amount of phytate phosphorus hydrolyzed.

6. The results are tentatively explained as being due to hydrolysis of the calcium and magnesium phytate at such a level in the digestive tract as to permit of the calcium being subsequently absorbed, and to a gradual adaptation of the body to a lowered intake of available calcium.

7. In two of the subjects, the retention of magnesium was at first lowered by the diet containing more phytate phosphorus. It was considered that this might be due to the precipitation of magnesium by the phytic acid. Later, while the subjects were still on this regime, magnesium retention improved, and changed but slightly when the phytate phosphorus of the diet was lowered.

8. The retention of total phosphorus was at first lowered in two of the subjects by the addition of phytate phosphorus to the diet, but subsequently improved. It improved still further in all subjects when the phytate phosphorus of the diet was reduced.

9. The retention of iron was virtually the same with high and low phytate-phosphorus diets.

10. Cereal diets rich in phytate phosphorus and low in calcium are commonly consumed in various parts of the world; consideration of what must occur under these conditions, together with an examination of the small amount of relevant experimental evidence available, lends a considerable amount of support to the conclusions reached in this paper.

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β-Glucuronidase and Cell Proliferation*

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After repeated feeding of menthol to mice, Fishman (1940) obtained results which, on statistical examination, showed an increase in β-glucuronidase activity in liver, spleen and kidney, as compared with organs from untreated animals. Similar results were obtained in dogs fed with borneol. Glucuronidase in uterus and other sex organs was unaffected by menthol and borneol. In Fishman's own interpretation of these important experiments, β-glucuronidase is assumed to be responsible for glucuronide synthesis in the body. A synthetic role for the enzyme has, however, still to be demonstrated, its physical properties and distribution in the body having been studied solely by means of its hydrolytic action on conjugated glucuronides. Since menthol and borneol have been proved to be excreted as the glucuronides in, e.g. the dog, and may conceivably behave in the same way in the mouse, Fishman suggested that in his experiments he was measuring adaptation by glucuronidase in response to the presence of excess substrate for its hypothetical synthetic action. Later this theory was extended to explain the elevation in uterine glucuronidase observed after administration of oestrogens to ovariectomized mice (Fishman & Fishman, 1944; Fishman, 1947). Oestrogens did not affect the enzyme in liver, spleen and kidney, and the additional assumption was required, and made, that the enzyme is specific in its synthetic action, according to its source, for different groups of substrate. No such specificity was, however, observed in its hydrolytic action *in vitro*, menthol glucuronide being used throughout in the assay of uterine glucuronidase under conditions found to be optimal for hydrolysis by spleen preparations.

Fishman determined the activity of his enzyme extracts by measuring, by means of its reducing power, glucuronic acid liberated from menthol glucuronide (Fishman, 1939). Sources of error in this procedure, arising largely from its lack of specificity, have been pointed out by other authors (Graham, 1946; Levvy, 1946, 1948), and have led to the development of more satisfactory methods of assay (Talalay, Fishman & Huggins, 1946; Kerr, Graham & Levvy, 1948).

Using phenol glucuronide as substrate in the assay of glucuronidase (Kerr *et al.* 1948), an attempt was made to confirm Fishman's findings (1940) with menthol. Within 24 hr. of a single intraperitoneal injection of L-menthol into mice, there was a marked rise in glucuronidase activity in liver, but not in spleen and kidney. Liver damage was observed and confirmed histologically, and it was subsequently shown that a rise in β-glucuronidase in liver or kidney, depending upon the organ or organs attacked, followed administration of a variety of toxic agents to mice. A more extensive examination of the action of menthol revealed, in addition to the effect on liver, delayed damage to kidney, followed by an increase in glucuronidase activity in this organ also. An increase in the glucuronidase activity of an organ was found, in general, to be associated with active cell proliferation provoked by injury, rather than with the injury itself, and high values were seen in the livers of adult mice after sub-total hepatectomy, and in the liver, spleen and kidneys of infant mice.

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* Preliminary accounts of parts of this work have been published elsewhere (Kerr & Levvy, 1947; Kerr, Levvy & Campbell, 1947), and the principal findings were described in a paper read to the Biochemical Society on 27 September 1947 (Levvy, Kerr & Campbell, 1948).

EXPERIMENTAL AND RESULTS

Enzyme assay. In the assay of kidney glucuronidase it was assumed that the conditions for optimum hydrolysis of phenol β-D-glucuronide would be the same as those previously found to hold for spleen and liver preparations (Kerr *et al.* 1948). All preparations of the enzyme were diluted to final volumes giving readings of 20–40 μg. phenol in the assay, after correction for blanks. The results are shown in the tables and figures in terms of glucuronidase units (G.U.)/g. moist tissue, where 1 G.U. liberates 1 μg. phenol in 1 hr. from 0·015 M-phenol glucuronide at 38° and pH 5·2. The standard error is given wherever possible. Although frequently based upon too small a group of animals to have any statistical value, it shows the variation in the individual figures in a convenient form.

Histology. Portions of organs from animals used for enzyme assay, or whole organs from other animals treated similarly, were fixed immediately in Susa and taken in the usual way through the ethanols to a mixture of chloroform and cedarwood oil, and finally cleared in pure cedarwood oil. After embedding in paraffin wax, sections were cut at 8 μ. and stained with Mayer's haematoxylin and eosin. The distribution of fat was studied in frozen sections, prepared from tissues rapidly fixed by heat in formol saline, and stained with haematoxylin and Sudan III.

Damage, repair and cell division are shown in the tables by an arbitrary system of + signs. In the case of damage, + indicates that while definite it was neither severe nor extensive, and + + + that it was at its greatest for the toxic agent in question. The course of repair is measured likewise, + + + indicating that replacement of damaged tissue by normal cells is practically complete. Under cell division, an estimate is given of the number of mitotic and amitotic figures and hyperchromatic nuclei *in excess of normal.* No histological findings are given for spleen since deviations from normal could never be distinguished in this organ.

Normal mice and vehicle controls. Average values for β-glucuronidase in each organ were the same for normal adult mice (30–40 g.) of both sexes and drawn from three different colonies, and all the results are grouped together in the tables. Spleen showed greater variation in its normal glucuronidase activity than did liver or kidney. Intraperitoneal injection of relatively large volumes of 0·9 % sodium chloride solution, olive oil or nut oil (the vehicles used for administration of toxic agents) had no effect on glucuronidase in any of the three organs examined after an interval of 1–2 days. These results are not shown. The relatively small number of experiments in which nut oil was used as a vehicle, olive oil being unobtainable, are included in the tables with those done with the latter as medium.

Effects produced by a single injection of L-menthol. Intraperitoneal injection of L-menthol (Table 1) caused a rapid rise in liver glucuronidase activity, reaching a maximum after 24 hr. and persisting for 7 days. Greatest liver damage was observed after 24 hr., but repair processes were not perceptible at this time. After 14 days, repair was almost complete and the enzyme level had returned to its original value, although cell division still seemed to be slightly in excess of normal. In the first 24–48 hr., kidney was normal in structure and in its enzyme activity, but after 3 days damage was evident and the figure for glucuronidase had risen after 7 days. At the end of 14 days this organ was normal in all respects. No effect of menthol on spleen glucuronidase was observed at any stage. Sex did not influence the results obtained with liver and kidney.

Mills (1947) found beef spleen glucuronidase to consist of two fractions with slightly different pH optima for the hydrolysis of menthol glucuronide, and the pH activity curves for hydrolysis of phenol glucuronide by enzyme from mouse spleen and liver (Kerr *et al.* 1948) had subsidiary peaks at pH 4·5. An experiment was done in which spleen, liver and kidney glucuronidase activities, 24 hr. after injection of menthol, were compared with normal at pH 4·5 instead of 5·2. The change in pH had no appreciable effect on the results compared with those shown in Table 1.

No details of the toxic action of menthol could be found in the literature. A brief description of the changes seen in liver and kidney may be of interest. In the liver, the first deviation from normal was cloudy swelling, followed by fatty change and necrosis surrounding the central vein and extending about a third of the way into the lobule. The nuclei showed hypertrophy and hyperchromatism, many

Table 1. *Changes in β-glucuronidase and histological findings after injection of mice with L-menthol*

(333 mg. Menthol/kg. injected intraperitoneally in olive oil. Average enzyme activity and standard error expressed as G.U./g. moist tissue (see text). Number of animals in group shown by figures in brackets.)

		Liver				Kidney			
Interval (days)	Spleen enzyme	Enzyme	Damage	Cell division	Repair	Enzyme	Damage	Cell division	Repair
Untreated	636±70 (23)	273±13 (23)	—	—	—	363±24 (11)	—	—	—
0·125–0·5	720±63 (9)	467±24 (9)	—	—	—	381±40 (9)	—	—	—
1	690±41 (3)	823±135 (3)	+ + +	0	0	285±46 (3)	0	0	0
2	738±86 (3)	884±74 (6)	—	—	—	344±71 (3)	—	—	—
3	903±208 (3)	953±39 (3)	+ +	+ + +	+	—	+ +	0	0
7	646±86 (6)	775±46 (7)	+	+ +	+ +	603±52 (7)	0	+	+ + +
14	600±14 (3)	318±17 (3)	+	+	+ +	337±23 (3)	0	0	+ + +

binucleate cells appeared (amitotic division), and at a later stage mitotic division became evident. The Küpffer endothelial cells were swollen. In the case of kidney, the damage was not severe, being confined to the distal portions of the convoluted tubules and to some glomeruli, the endothelium of which was swollen and in places necrotic. Intraperitoneal injection of mice with large doses of menthol (about 0·7 g./kg.) caused prolonged depression of the respiration and unconsciousness. No attempt was made to determine the lethal dose.

Repeated administration of L-*menthol and* L-*menthol β*-D-*glucuronide*. Results for β-glucuronidase in spleen, liver and kidney, after intraperitoneal injection of mice with L-menthol or its glucuronide twice or thrice daily for varying periods (Table 2), were similar to those obtained after a single injection of L-menthol. The glucuronide was injected as a neutral solution in 0·9 % sodium chloride solution and menthol itself as a solution in olive oil. (For the preparation of neutral solutions of acid compounds in 0·9 % sodium chloride solution, see Chance, Crawford & Levvy, 1945.) Repeated oral administration (Odell, Skill & Marrian, 1937) of menthol produced an increase in liver glucuronidase activity of the same order as the injections, except in mice receiving a total dose of 9·3 g./kg. in which the rise was barely perceptible. The latter was, however, as great as that obtained by Fishman (1940) and proved to be statistically significant ($P = 0{\cdot}01$). This experiment was carried out exactly as described by Fishman except that three of the mice were given a solution of menthol in olive oil instead of an emulsion in soap solution. The change in vehicle had no effect on the response of the enzyme, and only solutions in oil were used in the other feeding experiments (total dose 1·2 and 2·0 g./kg.). No histology was done in the experiments listed in Table 2, except in the case of menthol glucuronide, which produced changes similar to those seen after a single injection of menthol.

Table 2. *Changes in β-glucuronidase after repeated administration of* L-*menthol and* L-*menthol β*-D-*glucuronide*

(Results expressed as in Table 1)

Agent and mode of administration	Total dose (g./kg.)	Interval after 1st administration (days)	Average enzyme activity and S.E. (G.U./g. moist tissue)		
			Spleen	Liver	Kidney
Untreated	—	—	636 ± 70 (23)	273 ± 13 (23)	363 ± 24 (11)
L-Menthol, orally	1·2	3	843 ± 185 (3)	741 ± 146 (3)	—
	2·0	1	—	895 ± 77 (6)	—
	9·3	5	499 ± 48 (6)	369 ± 40 (6)	260 ± 2 (2)
L-Menthol, intraperitoneally	0·8	2	254 ± 38 (3)	1149 ± 136 (3)	—
	1·2	3	599 ± 42 (6)	869 ± 58 (6)	—
L-Menthol β-D-glucuronide, intraperitoneally	2·3	1·5	995 ± 95 (3)	1104 ± 222 (3)	295 ± 13 (3)

Changes produced by single injections of a variety of substances. The changes in β-glucuronidase activity and histological findings in liver and kidney after injection of various substances, some of them known liver or kidney poisons, are summarized in Table 3. Spleen was also examined in these experiments. Since any changes in glucuronidase in this organ were relatively small, with wide variation in individual figures, the results are not shown.

Subcutaneous injection of carbon tetrachloride in olive oil caused severe fatty degeneration and early necrosis in liver within 24 hr. After 3 days, damage was extensive, but repair processes had commenced, and after 7 days repair was far advanced. A marked increase in liver glucuronidase activity occurred within 24 hr., and this was maintained for 7 days. There were no marked pathological changes in kidney at any stage, nor was there any rise in glucuronidase in this organ. Intraperitoneal injection of carbon tetrachloride (0·5–2 g./kg.) produced a change in liver glucuronidase similar to that already described for subcutaneous injection.

Mercuric nitrate given subcutaneously in 0·9 % sodium chloride solution had no very marked effect on liver, but produced severe cortical necrosis with hyaline casts in kidney within 24 hr. Kidney glucuronidase activity showed no rise at this stage, but after 3 days, by which time repair was practically complete, it was more than twice its normal value.

The changes in liver after subcutaneous injection of chloroform in olive oil resembled those produced by carbon tetrachloride. Kidney, however, showed an interesting sex specificity in the response of the enzyme to chloroform. In agreement with the observation of Eschenbrenner (1944), this compound was found to cause renal necrosis in male, but not in female mice. The rise in kidney glucuronidase activity, which was confined to male mice, was not seen in the early stages of the damage, but was evident after 8 days, by which time repair was extensive. An increase in liver glucuronidase

Table 3. *Effects of various agents on liver and kidney*

(Results expressed as in Table 1)

Agent and dose	Interval (days)	Sex	Liver				Kidney			
			Enzyme	Damage	Cell division	Repair	Enzyme	Damage	Cell division	Repair
None	—	♀, ♂	273±13 (23)	—	—	—	363±24 (11)	—	—	—
Carbon tetrachloride (5·3 g./kg.)	1	♂	1138±148 (3)	+ + +	0	0	139±9 (3)	+ (?)	0	0
	7	♂	927±48 (4)	+	+ + +	+ +	323±45 (4)	0	0	0
$Hg(NO_3)_2$ (20 mg./kg.)	1	♂	436±28 (2)	+	0	0	208±42 (3)	+ + +	0	0
	3	♂	469±29 (5)	0	+	0	808±61 (5)	0	+	+ + +
Chloroform (2 g./kg.)	1	♂	939±90 (7)	+ + +	+ +	0	194±16 (6)	+ + +	0	0
	8	♂	711±34 (3)	+	+ +	+ +	628±119 (3)	+	+ +	+ +
	1	♀	583±27* (3)	—	—	—	251±18 (3)	0	0	0
	8	♀	608±44 (3)	+	+ +	+ +	274±15 (3)	0	0	0
Yellow phosphorus (7·5 mg./kg.)	2	♂	91±23 (3)	+ + +	0	0	338±35 (3)	+ (?)	0	0
	5	♂	744±37 (3)	+ +	+ +	+	462±89 (3)	0	0	0
	10	♂	429±80 (3)	0	0	+ + +	309±32 (3)	0	0	0
Sulphathiazole (43 g./kg.)	3	♂	460±29 (3)	+	0	0	368±13 (3)	0	0	0
Pregnanediol (333 mg./kg.)	1·7	♀	287±65 (3)	0	0	0	321±64 (3)	0	0	0
†Pregnanediol β-D-glucuronide (800 mg./kg.)	1·7	♀	241±36 (3)	—	—	—	264±19 (3)	—	—	—
Ether (40 min. deep anaesthesia)	1·75	♀, ♂	265±30 (4)	0	0	0	309±63 (3)	0	0	0
Sodium sulphapyridine monohydrate (18–36 g./kg.)	2	♀, ♂	327±17 (7)	+ +	+	+	362±11 (7)	+ (?)	0	0

* Results for glucuronidase in liver obtained after intraperitoneal injection of 0·5 g. chloroform/kg.

† Pregnane-3(α):20(α)-diol glucuronidic acid free from pregnane-3(α)-ol-20-one glucuronidic acid (Sutherland & Marrian, 1947).

activity was observed after injection of as little as 0·2 g. chloroform/kg. subcutaneously.

Yellow phosphorus, injected subcutaneously in olive oil, had no marked effect on kidney, but produced profound and extensive changes in liver (congestion, fatty degeneration and necrosis). From the results of experiments dealt with above it will have been noted that there may be no rise in glucuronidase activity in an organ when damage is at its height. In the case of phosphorus, there was an unmistakable initial drop in the activity of the enzyme, to one-third of its normal value. When, at the end of 5 days, repair was well under way, the enzyme level showed the usual increase, only to fall again when repair was complete.

Of the remaining substances listed in Table 3, ether and pregnanediol produced no pathological changes and had no effects on glucuronidase in either liver or kidney. Pregnanediol glucuronide resembled the parent compound in its effects on the enzyme, but was not examined for histological effects. Ether was given by inhalation, and pregnanediol and its glucuronide were injected intraperitoneally as suspensions in olive oil. Sulphathiazole caused cloudy swelling in liver after subcutaneous injection of a very large dose as a neutral solution in 0·9% sodium chloride solution. There was a small, but significant ($P=0{\cdot}05$–$0{\cdot}02$) rise in liver glucuronidase activity. This compound had no effect of any kind on kidney. Sodium sulphapyridine, given in the same way as sulphathiazole, caused fatty degeneration and necrosis in liver, accompanied by some cell proliferation, but without appreciable change in the enzyme level. In some animals there was slight damage to the kidneys, again without any rise in glucuronidase activity.

Uranyl acetate. Results for glucuronidase in liver and kidney after subcutaneous injection of mice with varying doses of uranyl acetate in 0·9% sodium chloride solution (Fig. 1) illustrate the point that increasing the dose of a toxic agent may retard the rise in glucuronidase activity in the early stages of poisoning, and may even cause an initial drop in the enzyme level. Each point in the figure is an average for a group of three male mice, killed 2 days after injection. Severe tubular 'nephrosis' was noted at this stage with all four doses of the toxic agent. Cell proliferation could also be seen after all but the largest dose, becoming more marked as the dose fell. In the case of liver, the histological findings were more difficult to interpret as the damage, which was mainly subcapsular, was transitory and rapidly succeeded by intensely active cell proliferation. Only the latter response was observed after injection of the smallest dose of uranyl acetate. In general, however, damage was greater and repair processes slower to appear as the dose was increased. Ten days after injection of the smallest dose of uranyl acetate repair was finished in both liver and kidney, and their enzyme levels had returned to normal. No change in spleen glucuronidase was produced by uranyl acetate.

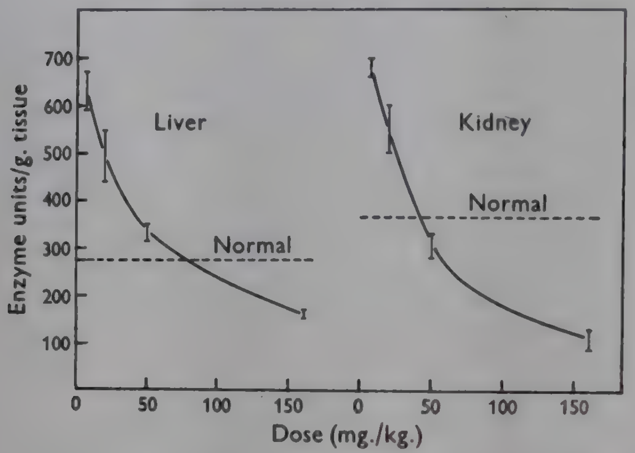

Fig. 1. Liver and kidney glucuronidase activity 2 days after subcutaneous injection of mice with varying doses of uranyl acetate. Mean ± S.E. shown for each point.

The possibility that in a severely damaged organ an apparently normal value for glucuronidase may be observed at a certain stage, even though cell proliferation may have commenced, probably explains the fact that only small rises in the liver enzyme were observed after prolonged feeding of menthol (total dose 9·3 g./kg., Table 2) or subcutaneous injection of sodium sulphapyridine (Table 3).

Phenylarsenoxide. Results obtained with this compound (Fig. 2) show the changes in liver and kidney glucuronidase activity at various stages in different degrees of poisoning. Phenylarsenoxide was injected subcutaneously as a neutral solution in 0·9 % sodium chloride solution. Each point in the figure is an average for a group of three male mice, except in the case of the 1 day figures with the larger dose, which are both based on six results. Phenylarsenoxide caused peripheral lobular necrosis and fatty degeneration in liver, and diffuse nephritis in kidney. Damage to both organs was intense 1 day after injection of 1 mg./kg., with no signs of repair. After 3 days, repair processes had become evident, and replacement of damaged tissue was well advanced after 5 days. At the end of 10 days, kidney was entirely normal and liver repair was almost complete. With the smaller dose (0·2 mg./kg.), cell proliferation was marked in both organs after 1 day, and repair was far advanced after 4 days. Phenylarsenoxide had no effect on spleen glucuronidase, and the histological changes produced in liver and kidney were similar for both sexes.

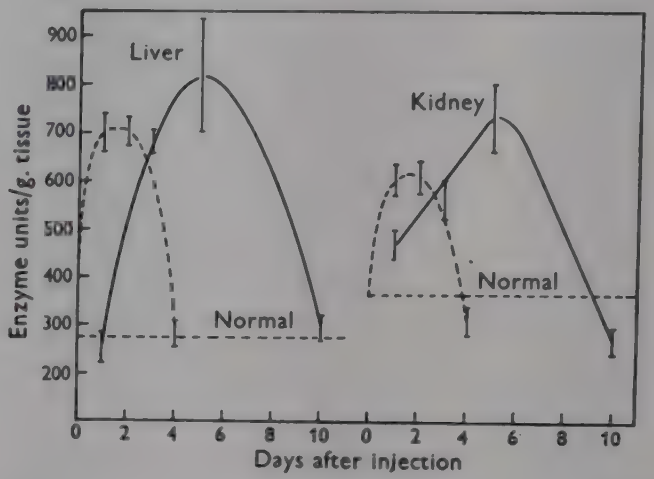

Fig. 2. Liver and kidney glucuronidase activity at varying periods after subcutaneous injection of mice with phenylarsenoxide. ——, 1 mg./kg.; — —, 0·2 mg./kg. Mean ± S.E. shown for each point.

Effects of various substances on the enzyme in vitro. All the substances examined for their effects on β-glucuronidase activity *in vivo*, with the exception of pregnanediol glucuronide, were tested for their effect on the assay *in vitro* in a concentration of 0·1 % (w/v). A solution in the medium used for injection was added to the citrate buffer and shaken vigorously. In no case did the presence of the agent in the incubation mixture affect the activity of β-glucuronidase from normal mice.

Infant mice and partially hepatectomized mice. As shown in Table 4, glucuronidase activity in spleen, liver and kidney was much higher in young mice, ranging in age from 1 to 15 days, than in normal adults. The remaining lobes of liver in adult mice (male and female), 3–8 days after sub-total hepatectomy, were hypertrophied. The glucuronidase level was high and cell proliferation was very active. In preparing the animals, 60 % of the liver was removed by cautery or ligature under ether anaesthesia, the whole operation taking less than 10 min. There was no difference in the final result between the alternative surgical techniques, nor at the various times of examination. One animal (result omitted from Table 4) was comatose and apparently about to expire when killed 3 days after operation. As expected, the remaining fraction of the liver showed no increase in weight, in the glucuronidase activity nor in cell proliferation.

Table 4. *β-Glucuronidase after sub-total hepatectomy and in infant mice*

(Results expressed as in Table 1)

Age (days)	Treatment	Average enzyme activity and S.E. (G.U./g. moist tissue)		
		Spleen	Liver	Kidney
Adult	None	636±70 (23)	273±13 (23)	363±24 (11)
1*	None	5100 (3)	1370 (3)	881 (3)
5*	None	2670 (2)	1294 (2)	702 (2)
5*	None	3820 (2)	1432 (2)	883 (2)
13*	None	1521 (2)	2218 (2)	606 (2)
15	None	5169±2820 (3)	1239±49 (3)	727±108 (3)
Adult	Partial hepatectomy 3–8 days previously	—	1046±88 (10)	—

* Each organ pooled before enzyme assay.

DISCUSSION

Our own results with menthol confirm Fishman's (1940) findings in so far as the enzyme in liver and kidney is concerned. It seems, however, that Fishman suppressed repair processes by overdosage with menthol, and thus obtained a rise in glucuronidase which was only a small fraction of that provoked by the first doses of the compound. No explanation can be offered for our failure to observe the rise in the activity of spleen glucuronidase in menthol-treated mice reported by Fishman, unless the discrepancy has its origin in the wide variation in its glucuronidase level normally shown by spleen. This variation one might expect if glucuronidase activity is a measure of the amount of cell proliferation in progress.

The present work shows that the effects of menthol on β-glucuronidase activity *in vivo* bear no relation to its glucuronidogenic property, but are secondary to its hitherto unsuspected toxic action on liver and kidney. It seems impossible that chloroform, carbon tetrachloride, mercuric nitrate, phosphorus or uranyl acetate should give rise to a glucuronide in the body, and yet all these substances have been found to cause striking changes in glucuronidase activity. Of other substances which caused a rise in glucuronidase, phenylarsenoxide could conceivably form a derivative conjugated with glucuronic acid, and evidence has been obtained that sulphathiazole is partially excreted in rabbits as the glucuronide of a hydroxy derivative (Thorpe & Williams, 1940). In spite of the very large dose injected, sulphathiazole caused only a relatively small rise in glucuronidase activity in mice, and this was confined to liver. No change in glucuronidase was observed after injection of two compounds which are known to be glucuronidogenic, pregnanediol (Venning & Browne, 1936) and sodium sulphapyridine (Scudi, 1944). It should be pointed out that a change in experimental conditions might reveal an effect of sulphapyridine on glucuronidase in liver, since it produced some damage in this organ. The effect of menthol glucuronide on liver glucuronidase was due presumably to menthol liberated by the enzyme initially present. Fishman's theory (see p. 462) provides no explanation for a change in the enzyme brought about by administration of a compound already conjugated with glucuronic acid.

On the basis of the experiments described above it is not possible to decide whether β-glucuronidase is actually concerned in cell proliferation, or whether the increases in activity observed merely reflect an increase in metabolic activity. It is interesting to note, however, that the rise in the enzyme level occasionally slightly preceded the first appearance of cell division which was definitely in excess of normal. Whatever the cause of the parallelism between the glucuronidase activity in an organ and the amount of tissue growth in progress, it provides a straightforward explanation of the changes in the enzyme in liver and kidney which follow administration of menthol and other substances to mice. It seems possible that the same explanation can be applied to the effect of oestrogens on uterine glucuronidase (Fishman & Fishman, 1944; Fishman, 1947), and to a recent observation (Fishman & Anlyan, 1947), which suggests that in some cases of human carcinoma the tumour contained more glucuronidase than the corresponding normal tissue. The possible bearing of our results with carbon tetrachloride on the finding (Pincus & Martin, 1940) that, in liver poisoning produced by this compound, the physiological activity of oestrone is enhanced is of interest.

It is no longer necessary to speculate on the probable role of glucuronidase in the body in order to explain the changes in activity produced by extrinsic agents. The citation by Fishman (1947) of the work of other authors in support of his contention that the enzyme acts synthetically, however, makes it necessary to consider their results from this angle. Florkin, Crismer, Duchateau & Houet (1942) obtained evidence for the condensation of glucuronic acid with borneol in the presence of β-glucuronidase, but the percentage conjugation was very small under extreme conditions, and they concluded: 'Quant à savoir si cette synthèse enzymatique correspond au mécanisme réalisé *in vivo*, c'est évidemment une autre affaire.' In the work of Lipschitz & Bueding (1939) and Crépy (1946) on the formation of conjugated glucuronides by surviving liver slices, there is no suggestion that the enzyme concerned is β-glucuronidase. De Meio & Arnolt (1944), who studied conjugation of phenol by surviving tissue slices, found that glucuronic acid reversed the inhibition of this process produced by iodoacetate. They also found that feeding phenol and borneol to rats increased phenol conjugation by liver and kidney *in vitro*. Their results are difficult to interpret since it is known that phenol may be conjugated with either sulphuric or glucuronic acid. Even if De Meio & Arnolt are correct in thinking that, contrary to the views of Lipschitz & Bueding (1939), glucuronides are formed by direct condensation of the 'aglucurone' with free glucuronic acid, there is no reason to believe that β-glucuronidase is responsible. With regard to De Meio & Arnolt's second finding, there is, in view of our own work, no need to postulate adaptation by the enzyme or enzymes responsible for conjugation of phenol, since both phenol and borneol may have caused liver and kidney damage in their experiments. Results obtained by Bueding & Ladewig (1939) in studying the effect of chloroform poisoning in guinea pigs on glucuronide synthesis by liver slices are of interest in this connexion. Not only did the liver slices from the poisoned animals show the usual increase in glucuronide synthesis on addition of lactate, but they were apparently more active in forming borneol glucuronide than slices from normal animals. The latter aspect of their results is not touched upon by the authors. While there is thus some evidence to suggest that, following damage to an organ, there may be an increase in its ability to form conjugated glucuronides, it is at present impossible to say whether or not this is due to the rise in β-glucuronidase activity observed when repair is in progress, nor is it certain that the glucuronides are formed directly from free glucuronic acid.

SUMMARY

1. The effect of menthol administration to mice in increasing the β-glucuronidase activity of liver and kidney is due to its toxic action on these organs. The rise in enzyme activity is associated with an increase in cell proliferation following injury. Menthol had no effect on spleen glucuronidase.

2. Among other substances examined, the following caused changes in glucuronidase in liver or kidney in an analogous fashion to menthol: chloroform, carbon tetrachloride, mercuric nitrate, yellow phosphorus, phenylarsenoxide, uranyl acetate, menthol glucuronide and sulphathiazole. The effect of chloroform on kidney glucuronidase was confined to male mice.

3. Livers from adult mice after sub-total hepatectomy, and spleens, livers and kidneys from infant mice showed high glucuronidase activities.

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The Use of Radioactive Isotopes in Immunological Investigations

1. PRECIPITIN REACTIONS WITH OVOVITELLIN CONTAINING P^{32}

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Analysis of the specific precipitates obtained from antigen-antibody mixtures can help to throw light on many problems of immunology; for example, the quantitative relationship between antigen and antibody in the precipitin reaction, the behaviour of 'inhibitory' substances, and the relative avidities of closely related antigens. Total N determinations on the antigen and on the specific precipitates have been used to determine the antibody/antigen ratio (Heidelberger & Kendall, 1929; cf. Heidelberger, 1939), and this method is of particular value when polysaccharide antigens containing little or no N are used. Use has also been made of antigens containing readily determinable elements or groups which are absent from the antibody. Amongst such 'labelled' antigens are haemoglobin (Wu, Cheng & Li, 1929), iodoalbumin (Wu, Sah & Li, 1929), haemocyanin (Hooker & Boyd, 1936) and proteins conjugated with dyes (Heidelberger & Kendall, 1930, 1935). For a somewhat different purpose N^{15} has been used as a tracer for investigations on the formation and the circulation of antibodies (Schoenheimer, Heidelberger, Rittenberg & Ratner, 1941; Schoenheimer, Ratner, Rittenberg & Heidelberger, 1942; Heidelberger, Treffers, Schoenheimer, Ratner & Rittenberg, 1942).

In the present investigation an attempt has been made to use antigens containing radioactive tracer elements for serological investigations. These elements can be determined in extremely minute amounts, and the determinations are not affected by the presence of relatively large amounts of the corresponding non-radioactive element. Two convenient isotopes for this type of work are S^{35} and P^{32}.

For the preparation of radioactive antigens the following methods were considered: (i) conjugation of a natural protein with a simple organic substance containing the radioactive element; (ii) direct phosphorylation of natural proteins or of dephosphorized casein, using phosphoryl chloride prepared from radioactive P; (iii) a biological preparation of a protein containing the particular element whose radioisotope is to be used (e.g. vitellin containing P^{32}, haemoglobin containing Fe^{55} or Fe^{59}). With regard to the first method, Berenblum & Wormall (1939), and Boursnell, Francis & Wormall (1946) have investigated the serology of proteins treated with $\beta\beta'$-dichlorodiethylsulphone, and Banks, Boursnell, Francis, Hopwood & Wormall (1946) have described the preparation of mustard gas sulphone-protein derivatives containing radioactive sulphur (S^{35}). We have recently used this S^{35}-containing conjugated antigen, and phosphorylated proteins containing P^{32}, for some preliminary serological investigations, and a brief account of the results has been given elsewhere (Boursnell, Dewey, Francis & Wormall, 1947). The present paper deals with the preparation of hen ovovitellin containing P^{32} and its use in quantitative precipitin reactions.

METHODS

Collection of radioactive eggs. In the first experiment, two hens (nos. 815 and 816) were each given an intramuscular injection in the right thigh of 50 microcuries (μC) of P^{32} (in the form of neutral sodium phosphate, in 1–1·5 ml. of 0·9% (w/v) NaCl solution). In the second experiment, hen 816 was given a single injection of 380μC, about 1 month after the first. The eggs were collected for 1 month after the first injection and worked up in small batches (1 or 2 eggs laid on the same day, for the first 12 days, and batches of 7 eggs thereafter). In the second experiment a vitellin preparation of relatively high radioactivity was obtained from the eggs laid on the 4th, 5th and 6th days following the injection.

Separation of vitellin. This was carried out essentially by the method of Calvery & White (1932). The separated and washed yolks were punctured, and the membrane contents allowed to flow out into 20 ml. of 10% (w/v) NaCl per yolk. As much lipid as possible was removed by extraction with ether containing 2% ethanol (6–8 extractions), and the vitellin in the saline solution was then precipitated twice with 20 vol. of tap water. (It was found that some samples of ether caused denaturation of the vitellin, rendering it insoluble in 10% NaCl, but freshly opened bottles of ether, sp.gr. 0·720 (British Drug Houses Ltd.), were satisfactory.) For most purposes the final product was dissolved in 10% NaCl to give a total vol. of 50 ml. per egg, and used in this form ('soluble' or lipovitellin).

In a few experiments, however, further removal of lipid was effected, either by the hot ethanol treatment of Calvery & White (1932) or by pouring the cooled NaCl solution into 5 vol. of a cooled 7 : 3 ethanol-ether mixture, centrifuging, washing twice with ethanol-ether and twice with water, all these operations being carried out at 0° to −5°. Both extraction processes gave products ('insoluble' vitellin) with approximately the same P/N ratio (about one half that of the lipovitellin), but both rendered the products insoluble in 10% NaCl. The product obtained by the cold ethanol-ether method was, however, soluble in saturated urea solution.

Preparation of radio-lecithin. The total ether extracts of the radioactive yolks were dried over anhydrous Na_2SO_4, evaporated to dryness, and the residues redissolved in dry ether. The radio-lecithin was separated from this solution by pouring into acetone and purified by redissolving in chloroform and reprecipitating with acetone. The final product was dried *in vacuo* over $CaCl_2$. In some serological tests radio-lecithin was added to an antiserum; this was effected by emulsifying the lecithin with normal rabbit serum, centrifuging and adding the supernatant fluid to the antiserum.

Radioactivity of the eggs. The main object of these experiments was the preparation of radio-vitellin, and at a later stage radio-lecithin, for immunological experiments, but a few measurements on the individual eggs, and on certain fractions of each egg, were made in order to obtain some idea of the rate of elimination of the injected P^{32} in various fractions of the egg. Hevesy & Hahn (1938) and Aten (1939) have studied the radioactivity of the lecithin fraction of the eggs of hens injected with P^{32}, but no specific information about the vitellin was available to us when our investigations were started. Radio-determinations were made on (*a*) the whole intact egg, (*b*) the whole yolk, (*c*) the separated total lipid (i.e. the ethanol-ether soluble fraction of the yolk) and (*d*) the separated lipovitellin. The values recorded for (*a*) and (*b*) do not, of course, represent the total radioactivity of the material concerned, but they serve as a rough comparison of the P^{32} content of the shells and yolks of different eggs and they give some idea of the rate of elimination of the injected P^{32} in these two fractions of the egg.

For the intact egg 'count', the egg was placed directly under the window of the Geiger-Müller counter. After the egg had been broken, the empty shell was, in some cases, roughly reconstituted into its original shape, and was found to have almost the same radioactivity as the original egg; calculations showed that the white of the egg had absorbed over 75% of the radiations from the yolk. The radioactivity of the white was usually so small that it contributed little to the activity of the intact egg. Thus the 'counts' for the intact egg are of value in giving an approximate relative value for the radioactivity of the shell.

The whole washed yolk (and in some cases the whole white) from each egg was placed separately in small glass crystallizing dishes and 'counted'. These values also are merely relative, and they should not be compared with the values for the intact egg or the more accurate figures for the separated lipids and lipovitellin; they merely serve to give an idea of the day-to-day variation in the radioactivity.

For the yolk lipid 'counts', samples of the total ethanol-ether extract from each batch, corresponding to 1/10th of an egg, were evaporated in 20 ml. porcelain crucibles which were then placed under the counter. Similar determinations were made on fractions (each equivalent to 1/50th of an egg) of the final NaCl solution of lipovitellin from each batch, the solution being evaporated on a brass sample plate, and radioactivity determinations made. Tests showed that the amounts of material present in the samples were not sufficient to lead to any significant loss of the P^{32} radiation by 'self-absorption', and since the dimensions, and geometrical arrangements relative to the counter windows, of the lipid and vitellin layers were approximately the same, a general comparison of the two sets of results seems justifiable.

Immunization. Rabbits were injected with non-radioactive vitellin, two rabbits being immunized with the 'soluble' and three with the 'insoluble'. In each case intraperitoneal injections of 5 ml. of a solution (or suspension) in 10% NaCl were given, containing about 3% of protein and 0·25% phenol (as a preservative), at intervals of 7–10 days. Precipitin tests were carried out on sera taken from the rabbits 7–10 days after the fourth and subsequent injections. Good antisera were obtained from both rabbits injected with 'soluble' vitellin and one injected with 'insoluble' vitellin; the other rabbits yielded weak antisera.

Precipitin tests. Antiserum diluted with an equal vol. of 20% NaCl (0·4 ml.) was mixed with 2·0 ml. of diluted antigen in a 15 ml. tapered centrifuge tube. (The antigen was previously centrifuged for 20 min., and dilutions were made with 10% NaCl to avoid precipitation of the vitellin by a reduction in the salt content.) After 3 hr. at 37°, with occasional stirring, the tubes were centrifuged for 20 min. and the supernatant fluids discarded. The inside of each tube was washed down with 6 drops of 10% NaCl, care being taken to disturb the precipitate as little as possible, and the tubes were centrifuged for a further 7 min. The washed precipitates were dissolved in 4 drops of water plus 4 drops of 0·5 N-NaOH, and the solutions transferred to brass sample plates (containing kaolin). Each tube was washed out three times with 3 drops of water each time, and the washings added to the sample plates, which were then placed on a warm water bath until dry. A control experiment, with exactly the same technique, was carried out with normal rabbit serum added to the antigen solutions. Any radioactivity observed in the control, which was appreciable only with high concentrations of antigen, was subtracted from the value obtained for the corresponding mixture of antigen and antiserum.

Total P *determinations.* These were made by King's (1932) modification of the Fiske & Subbarow (1925) method.

Radioactivity measurements. These were made with the Geiger-Müller counter system described by Banks (1946). Owing to the much greater penetrating power of the radiations from P^{32} as compared with those from S^{35}, however, it was not found necessary to incinerate the materials before they were 'counted'. The plates were of the usual pattern, but were fitted with disks of 2 cm. diameter to improve the geometrical arrangements of the sample relative to the counter window. To facilitate the spreading and drying, about 50 mg. of kaolin were first suspended in a few drops of 50% ethanol and this suspension evaporated on the plate. The solution to be 'counted' (up to 0·5 ml.) was dropped on the kaolin pad and the plate heated over a warm water bath; the dried sample was protected against damage by adding 10–15 drops of 1 : 50 collodion in ethanol-ether mixture and was then redried.

This simple evaporation method for the preparation of samples for P^{32} determinations on serological precipitates and other organic material containing the radio-isotope greatly facilitates the determinations. It is desirable that, where this method is used, checks be made by incinerating some of the material and separating the phosphate as magnesium ammonium phosphate for radioactivity determinations. This control procedure has been adopted in the work described in this paper and in all cases the simple 'evaporation' method has given essentially the same result as the digestion or incineration method.

RESULTS

The excretion of P^{32} *in various fractions of the eggs laid by hens injected with* P^{32}*-containing phosphate*

The total radioactivities of the shell, whole yolks, and lipid and lipovitellin fractions of the eggs laid by the two hens during the month following the injection of 50 μC of P^{32} into each hen are given in Figs. 1 and 2. Owing to normal variations between individual eggs, particularly those laid by different hens, the points do not always lie on a smooth curve; but it is quite evident that the radioactivity of the vitellin and lipid fractions of the yolks reached a maximum about the 4th day after the injection, remaining approximately constant for a further 6 or 7 days, and then decreasing rapidly. For most of our subsequent immunological work, eggs laid 4–11 days after the injection of P^{32} have been used for the preparation of radio-vitellin and radio-lecithin. At the maximum, each egg contained in the lipovitellin plus lipid about 1 % of the total P^{32} injected, with about two thirds of this in the lipid fraction. The activities given for the shells and the whole yolks are, as pointed out above, purely arbitrary values and have only relative significance.

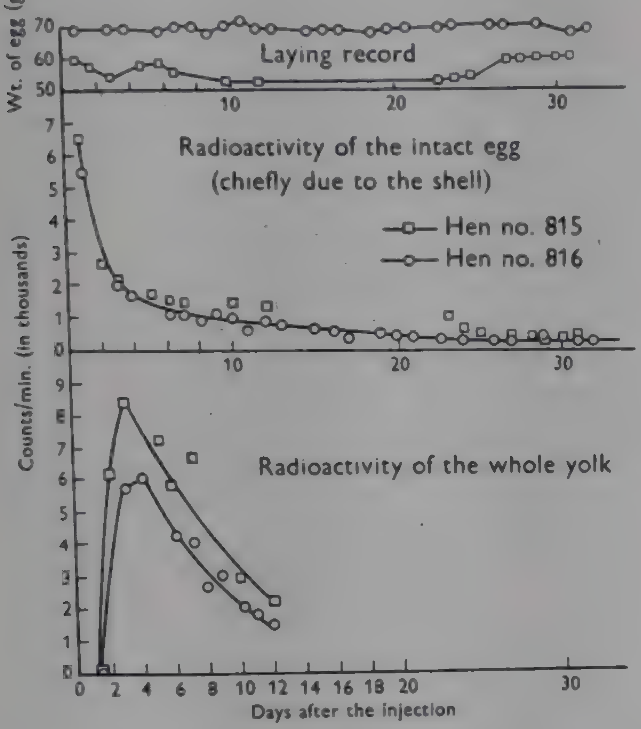

Fig. 1. Radioactivity of the intact egg (i.e. shell) and the yolk of eggs from hens injected with phosphate containing P^{32}. The values given in these figures are not absolute values, but merely give the relative activities of the shell (or yolk) from day to day (see text).

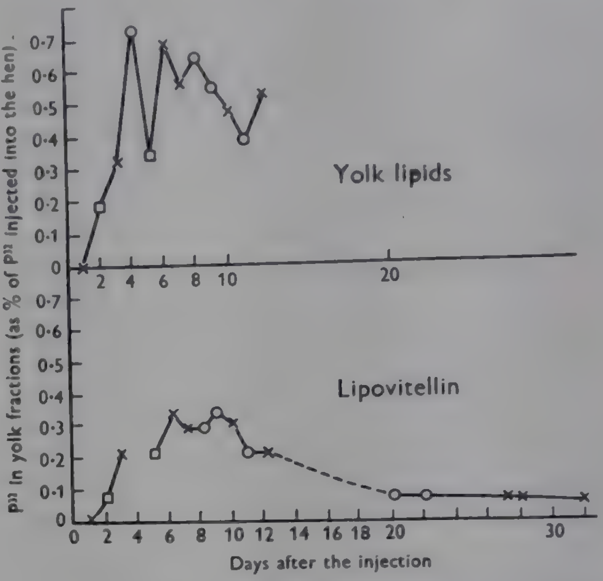

Fig. 2. Radioactivity of the yolk lipids and lipovitellin of eggs from hens injected with phosphate containing P^{32}. The values are expressed as activity/single egg. From the 1st to 12th day, each individual egg was examined; thereafter the eggs were grouped in batches of seven. Hen no. 815 —□—. Hen no. 816 —○—. Mixed yolks (815+816) —×—.

The determination of P^{32} *in vitellin-anti-vitellin precipitates*

Table 1 gives the results of determinations of the amount of antigen in the precipitates obtained by treating 0·20 ml. samples of a lipovitellin antiserum

Table 1. *Radio-*P *content of precipitates with 'radio'-vitellin and rabbit anti-vitellin serum*

(0·20 ml. of antiserum + 0·20 ml. of 20 % (w/v) NaCl were added to 2·0 ml. of 'soluble' radio-vitellin in 10 % (w/v) NaCl. Precipitates were centrifuged after 3 hr. at 37°. Control: normal rabbit serum instead of antiserum.)

		Radio-P precipitated			
Concentration of antigen (% protein)	Radio-P in 2 ml. antigen (μg.)	Rabbit antiserum* (μg.)	Control serum (μg.)	Average minus control (μg.)	As % of total antigen P
2·3	942	9·45, 12·4	13·4	0·0	0·0
0·37	157	30·9, 32·6	0·6	31·1	20
0·13	52·3	18·3, 18·3	0·5	17·8	34
0·042	17·4	6·34, 7·02	0·4	6·3	36
0·014	5·81	2·2, 2·1	0·0	2·1	37
0·0046	1·94	0·85, 0·61	0·0	0·7	37

* Duplicate experiments.

(no. 457) with 2·0 ml. samples of solutions containing radioactive lipovitellin. (Both lipovitellin and 'insoluble' vitellin injections produced antisera capable of precipitating lipovitellin.) It can be seen that, with the lower concentrations of antigen, approximately the same fraction (about 37 %) of the total P^{32} present in the antigen solution was precipitated in each case, down to extremely low concentrations of antigen. With moderate excess of antigen, the percentage of antigen P precipitated was less, while with a large excess none of the P^{32} was precipitated, thus confirming the visual observation in the precipitin test mixtures.

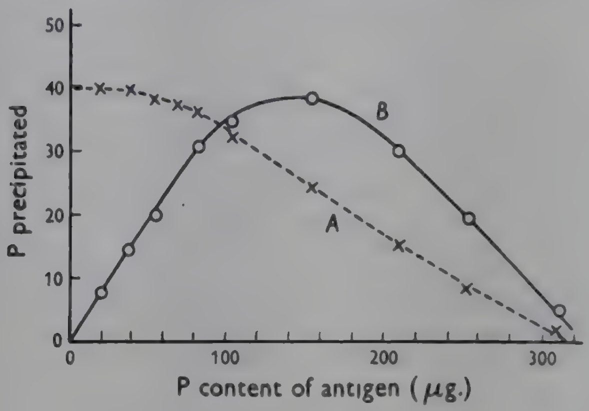

Fig. 3. P^{32} content of lipovitellin-antilipovitellin precipitates. *A*, percentage of total antigen P precipitated. *B*, total P precipitated (μg.). Antigen: 2·0 ml. radio-lipovitellin (P/N ratio = 0·14) in 10 % NaCl. Antiserum: 0·2 ml. rabbit antiserum to lipovitellin + 0·2 ml. 20 % NaCl. Precipitates: centrifuged after 3 hr. at 37° and washed once with 10 % NaCl.

In a further experiment a much smaller range of antigen concentration was covered, involving the use of antigen (radio-lipovitellin) solutions varying in strength from 0·05 to 0·74 %. This range covers the points of maximum precipitation and extends into the excess-antigen inhibition zone, under the conditions of our experiments. From the results of this experiment (Fig. 3) it will be seen that, with the lowest concentrations of antigen, there was again precipitation of about 40 % of the antigen P. Where there was the maximum amount of P^{32} in the precipitate, this represented about 25 % of the P^{32} in the antigen solution used.

The part played by lipids in the lipovitellin-antivitellin precipitin reaction

Since vitellin preparations, particularly the 'soluble' variety, contain considerable amounts of phospholipins, a few experiments have been made to determine whether these take part in the precipitin reaction. Varying amounts of radio-lipovitellin were added to measured volumes of antivitellin serum, four tubes being set up for each antigen dilution. After the mixture had been incubated and centrifuged, two tubes of each mixture were extracted three times with an ethanol-ether mixture to remove lipid matter. P^{32} determinations were made on the unextracted and the extracted precipitates and on the ethanol-ether extracts. These results (Table 2) showed that 70–80 % of the P^{32} in these specific precipitates was of phospholipid type (i.e. extractable with ethanol-ether). Since the lipovitellin preparations *A* and *B* used as antigens contained only 47 and 41 % respectively of their total P^{32} as phospholipin, there appears to have been preferential precipitation of much of the phosphatide by the antiserum. Parallel control tests made with an unrelated precipitin reaction (human serum plus anti-human serum) carried out in the presence of radio-lipovitellin gave precipitates containing no significant amount of P^{32}. It is concluded, therefore, that the preferential precipitation of phospholipin in the lipovitellin-antivitellin precipitates is not simply due to the mechanical carrying down of lipid by the precipitate.

Table 2. *The lipid* P *of lipovitellin-antivitellin precipitates*

(Precipitin reactions as in Table 1. Some of the precipitates were extracted three times at room temperature with 5 ml. of 7 : 3 ethanol-ether for 30 min. each time. The extracted precipitates were dissolved in dilute NaOH, and the ethanol-ether extracts were concentrated to small bulk. Both solutions were then transferred to sample plates for P^{32} determinations.)

		Radio-P content			
			Lipovitellin-antivitellin precipitate		
				Extractable by ethanol-ether	
Exp.	Concentration of antigen (%)	Antigen* (μg./2 ml.)	Total (μg.)	(μg.)	As % of precipitated P
1	0·377	157·5	37·7	26·9	71
	0·251	104·7	34·0	24·7	73
2	0·322	152·4	7·4	5·8	78
	0·214	101·6	11·3	9·0	80
	0·161	76·2	10·2	7·9	78

* 47 % of the P^{32} of the lipovitellin (preparation *A*) used as antigen in Exp. 1 was extractable with ethanol-ether, and the corresponding value for preparation *B* (Exp. 2) was 41 %. *A* and *B* had P/N ratios of 0·14 and 0·158, respectively.

In other experiments radio-ovolecithin has been added to mixtures of non-radio-lipovitellin and antivitellin. The specific precipitates formed in these mixtures contained very appreciable amounts of radio P. Further experiments are being made to determine whether this is simply due to interchange of lecithin between the solution and the antigen, or whether it is due to the direct combination of the radio-lecithin with the antivitellin or with the antigen-antibody complexes.

DISCUSSION

For the preparation of a 'labelled' protein which can be used as an antigen, the injection of P^{32} into hens and the separation of P^{32}-containing vitellin from the eggs laid by these hens has proved very satisfactory, with a reasonably good utilization of P^{32}. The injection of P^{32} is known to lead to the early appearance of the radio isotope in the various P-containing compounds of the hen and its eggs (Hevesy & Hahn, 1938; Aten, 1939; Chargaff, 1942*b*). In our experiments, the shell of the egg laid the day after the injection was highly radioactive, but that laid on the second day had only about half this activity; subsequently, the radioactivity of the shells fell to a relatively low level (Fig. 1). The whites of the eggs showed very little radioactivity, particularly after the first day or two, but the P^{32} content of the yolk lipids and the lipovitellin increased fairly rapidly up to a maximum at about 4 days after the injection, and then decreased slowly (Fig. 2).

These results indicate that the eggs laid 4–11 days after the injection of P^{32} into the hen serve best as sources of P^{32}-containing vitellin and lecithin. In one experiment, for example, about 2 % of the injected P^{32} was present in the lipovitellin and a little over 4 % in the yolk-lipids of the eggs laid during this period. Labelled vitellin and lecithin can thus be readily obtained with a specific radioactivity adequate for many immunological purposes, including the use of these compounds in microprecipitin tests and for some *in vivo* immunity experiments. In many of the precipitin tests described in this paper, rather large amounts of antigen solution were used, because of the relatively small amount of P^{32} available to us, but it is possible, by injecting more P^{32} and by adopting a special microprecipitin-test technique, to make reliable P^{32} determinations on precipitates from considerably less than 0·10 ml. of antigen. Although we used relatively large volumes of antigen solution for the tests, it should be remembered that some of our precipitates which gave 'counts' which could be measured accurately, were obtained from less than 0·05 ml. of the stock antigen solution (2·3 % radio-lipovitellin in 10 % NaCl solution).

Lipovitellin is not an ideal protein complex for serological investigations, partly because of its relative insolubility and partly because it contains a very considerable amount of bound phosphatide. The first difficulty can be overcome by adding NaCl, to give a concentration of 5–10 % (w/v), to all the solutions used for the precipitin tests; but the second problem cannot be solved so easily. The extraction of the firmly bound phosphatide with hot ethanol or with ethanol-ether mixtures in the cold renders the vitellin almost insoluble in water or salt solutions. It is then practically useless for precipitin tests, although it might be satisfactory for complement-fixation tests.

The immunological properties of vitellin have been studied by several authors. Wells (1909, 1911) showed, by anaphylactic experiments, that it is antigenic and that it is serologically distinguishable from ovomucoid and ovalbumin. Emmerich (1913) and Seng (1913) investigated the serological cross reactions between the yolk proteins of the hen's egg and those of the eggs of other birds, but as no separation of the vitellin was carried out, many of their results might well have been due to the other proteins present; hen egg-yolk livetin, for example, is antigenic, and immunological tests show that it is closely related to hen serum globulin (Jukes & Kay, 1932). Roepke & Bushnell (1936), using 'soluble' vitellin, obtained antisera which gave specific precipitation, and they were able to show that the serum of laying hens contains significant amounts of a protein similar to vitellin.

In our experiments immunization was effected with both the 'soluble' and the 'insoluble' vitellin, and both preparations produced good antisera in rabbits: since both these antisera gave precipitates with 'soluble' vitellin it is concluded that at least some of the reactive groupings in the antibodies are directed against groups of the protein molecule. Whether or not these antibodies can react with the lipid groups of lipovitellin is not known. The yolk phosphatides have been carefully studied in more recent years by Chargaff (1942 *a*, *b*; cf. also Chargaff, Ziff & Rittenberg, 1942) who found that removal of all the uncombined lipids with ether and repeated dialysis yielded a lipovitellin containing about 18 % of phosphatide and a total P content of 1·5 % (compared with 1·0 % in the ethanol-extracted product). Our 'soluble' vitellin preparations contained about the same percentage of phosphatide (15–22 %) as did the preparations of Chargaff, accounting for about half the P of the 'soluble' vitellin.

The specific precipitates obtained in our experiments contained considerable amounts of phosphatide, more in fact than did the lipovitellin used as antigen. In one experiment (e.g. Exp. 2, Table 2) about 80 % of the P^{32} of the precipitates was due to phospholipin, as against a figure of 41 % for the antigen. The combination (or perhaps adsorption) of the phosphatide by the antigen-antibody complex appears to be specific since a heavy precipitate formed between human serum proteins and their antibodies in the presence of radio-lipovitellin did not carry down any significant amount of phospholipin. It seems possible, therefore, that the lipovitellin antibodies have groups capable of reacting specifically with ovophosphatide. Precipitation might only occur in these mixtures when the antibodies have also reacted with the protein vitellin or with lipovitellin. It is however difficult to explain how the antibodies detach phosphatide from a lipoprotein complex, from which the phosphatide cannot be removed by ether extraction.

The nature of the linkage between ovophosphatide and the protein vitellin is not at all clear, and it would be of interest to know whether there is any significant serological difference between vitellin and lipovitellin. Removal of the ether-soluble substances from serum does not affect the true antigenic properties of the serum, i.e. its power to produce antibodies on injection, but specific precipitation does not occur when both antigen and antiserum are fully extracted with cold ethanol-ether (Hartley, 1925). Other specific precipitates vary in their lipid content, for whereas Horsfall & Goodner (1936) found 4–51 % of lipid in their pneumococcus polysaccharide precipitates, Marrack & Smith (1931) were unable to find any lipid in horse serum pseudoglobulin antigen-antibody precipitates.

The experiments described in the present paper were designed to indicate how a radio-isotopic tracer might be of value in one branch of immunochemistry. The results show that the tracer can be determined quite readily in precipitates obtained in ordinary precipitin tests. In these experiments no attempt was made to get maximum precipitation in all cases, e.g. by keeping the antigen-antibody mixtures in the refrigerator for several days, nor were any special efforts made in most of these experiments to purify the vitellin used as antigen, for it was not our desire to remove the firmly bound lipid from the vitellin. The results of other experiments, with fully extracted lipid-free vitellin, will be reported later.

SUMMARY

1. P^{32}-containing vitellin prepared from eggs laid by hens injected with P^{32}-containing phosphate has been used for immunological investigations.

2. P^{32} determinations have been made on precipitates obtained in ordinary precipitin tests to determine the amount of antigen in vitellin-antivitellin precipitates.

3. This isotopic tracer method, together with total-N determinations, will readily allow antigen/antibody ratios to be determined. Other possible uses of radio-isotopes for the study of immunochemistry are indicated.

4. Lipovitellin-antivitellin precipitates contained a much higher ratio of phosphatide P^{32}/protein P^{32} than did the lipovitellin antigens. This preferential carrying down of phosphatide by the precipitate is specific, and it does not occur in precipitin reactions unrelated to vitellin, e.g. in the precipitation of human serum proteins by their antibodies.

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N^1-Diethylsulphanilamide: a Reagent for the Colorimetric Estimation of Thyroxine

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The estimation of thyroxine in thyroid substance can be performed by biological assay, or by the determination of 'thyroxine iodine' in the hydrolyzed material. In the latter case the iodine content of the precipitate at pH 4·5, or of its butanol extract after treatment with a strongly alkaline aqueous phase, which retains the diiodotyrosine, can be expressed as 'thyroxine'. The methods have been reviewed by Harington (1933) and Salter (1940). On adapting the original butanol-extraction procedure of Leland & Foster (1932), as modified by Blau (1935), to artificially iodinated proteins, Reineke, Turner, Kohler, Hoover & Beezley (1945) compared their results with those of biological assay in the guinea pig and were led to conclude that the chemical method reliably accounted for the thyroxine present; the properties of any non-thyroxine component of the butanol layer would need to parallel closely those of thyroxine itself. From an examination of thyroid-gland preparations from different species, Parkes (1946) concluded that a fairly close relationship exists between acid-insoluble iodine (and total iodine) and the biological activity as determined in the *Xenopus* tadpole. On the other hand, Deanesly & Parkes (1945) found a very marked divergence from this correlation in the case of the acid-insoluble-iodine fractions from artificially iodinated proteins.

It would seem desirable to have an alternative method for the chemical assay of thyroxine, and we have been engaged in investigating the possibility of developing a specific colour reaction which could be quantitatively applied. Thyroxine gives a characteristic, though non-specific, red colour with nitrous acid, under special conditions, while the ninhydrin test is weakly positive (Harington, 1933). Recently Roche & Michel (1946) have reported a method for the quantitative estimation of thyroxine and diiodotyrosine based on the nitrous acid reaction. A further qualitative test was described by Komant (1930) in which a red or purple colour, transient in dilute solution, was produced by thyroxine in the presence of diazotized sulphanilic acid in alkaline solution. A colour, though much less intense, was also given by diiodotyrosine. Since both the 3′ and 5′ positions in thyroxine are occupied, the reaction mechanism, as inferred but not proved by Komant, presumably involves the replacement of the iodine at one of these points by the arylazo grouping. Thyroxine *O*-methyl ether does not react under these conditions (Loeser, Ruland & Trikojus, 1938). In examining this reaction further we have selected as reagent N^1-diethylsulphanilamide from a number of diazotizable amines using sensitivity, stability of the colour formed and the preference of the reaction product for the butanol phase in *n*-butanol-water mixtures as criteria. This last property was taken into account since, by transferring to butanol, thyroxine may be conveniently separated from contaminants, such as diiodotyrosine, prior to assay. The conditions of the reaction have also been studied with the object of increasing the thyroxine specificity and in order to standardize a procedure suitable for quantitative analysis.

EXPERIMENTAL

Preliminary studies

Selection of amine

Sulphanilic acid was chosen by Komant (1930) rather than its arsenic analogue, *p*-nitraniline or *p*-aminobenzoic acid, all of which gave, after diazotization, coloured reaction products with thyroxine in an alkaline medium (optimum pH 9–10). In Table 1 is listed a number of amines together with the results of our attempts to couple them with thyroxine, at pH 9–10, the test solutions being constituted as follows:

Thyroxine. A 0·1 % (w/v) solution in 0·004 N-NaOH.

Alkaline solution. NaOH (20 g.) and $NaHCO_3$ (28·8 g.) were dissolved in water and diluted to 1 l.

Diazo solution. In most cases the direct method of diazotization was used. The test amine (0·005 mol.) was dissolved or suspended in 9 ml. of 10 N-HCl, water was added to a volume of 100 ml. and 5 ml. of this solution or suspension was mixed with 25 ml. of 4·5% (w/v) aqueous $NaNO_2$ at 0° and allowed to stand for 10 min. Diazotization was checked by coupling a sample of the solution with α- or β-naphthol under conditions comparable to those used for the reaction with thyroxine. Only those amines whose diazotized solutions gave a strong positive reaction with

Table 1. *Reactions between thyroxine and various diazotized amines in aqueous solution* (pH 9–10) *and the solubilities, relative to the aqueous phase, of the coloured products in* n-*butanol*

No.	Diazotized amine	Colour		Transferred to *n*-butanol phase
		Thyroxine (0·1%)	Control (no thyroxine)	
1	Sulphanilic acid	Deep red	Yellow	–
2	Sulphanilamide	Red	Yellow	+
3	N^1-Ethylsulphanilamide	Rose-red	Pale yellow	+
4	N^1-Diethylsulphanilamide	Rose-red	Pale yellow	+
5	Sulphaguanidine	Red	Yellow	+
6	*m*-Sulphaguanidine	Red	Deep yellow	Partly
7	Sulphapyridine	Orange	Yellow	+
8	Sulphadiazine	Deep red	Yellow	–
9	Sulphathiazole	Red	Orange-yellow	–
10	Sulphamerazine	Yellow-red	Orange-yellow	–
11	1-Aminonaphthalene-4-sulphonamide	Brownish yellow	Brownish yellow	–
12	1-Aminonaphthalene-4-sulphonic acid	Brownish yellow	Brownish yellow	–
13	2-Aminonaphthalene-4:8-disulphonic acid	Red	Orange-yellow	–
14	4-Aminobenzoic acid	Red	Yellow	–
15	Ethyl 4-aminobenzoate	Red	Yellow	+
16	3:5-Dibromo-2-aminobenzoic acid	Yellow	Yellow	–
17	4-Aminoacetophenone	Brown-red	Yellow	+
18	*p*-Nitraniline	Red (purplish)	Deep yellow	+
19	2:4-Dichloroaniline	Red	Pale yellow	+
20	2:5-Dichloroaniline	Deep pink	Pale yellow	+
21	4-Diethylaminoaniline	Yellow	Yellow	–
22	*o*-Toluidine	Yellow	Yellow	–
23	*m*-Toluidine	Deep orange	Yellow	+
24	*p*-Toluidine	Orange	Yellow	+
25	2-*p*-Xylidine	Yellow	Yellow	–
26	4-*m*-Xylidine	Yellow	Yellow	–
27	*p*-Anisidine	Yellow	Yellow	–
28	4-Aminodiphenyl ether	Brownish yellow	Brownish yellow	–
29	4-Aminodiphenylamine	Brownish yellow	Brownish yellow	–

either of these two phenols are included in the table. The large excess of nitrite is as used by Komant (in our later studies with N^1-diethylsulphanilamide this reagent was appreciably reduced in quantity without affecting the reaction).

Procedure. The thyroxine solution (1 ml.) was mixed with the alkaline solution (1 ml.) and the solution of the diazotized amine (1 ml.) then added. A colour, when produced, was formed almost immediately. Redistilled *n*-butanol (1 ml.) was added after 3 min. and the tube was well shaken. A control (1 ml. of 0·004 N-NaOH) was treated in the same way.

Comments on Table 1. Although a systematic study of the relationship between amine structure and reactivity with thyroxine was not made, the presence of certain substituents in the position *para* to the amino group precluded reaction with the amino-acid but not with naphthol under the conditions used. If the colours in the control tube and in that containing thyroxine were comparable a negative reaction was recorded. This occurred when the *p*-substituent was $—N(CH_3)_2$, $—OCH_3$, $—OC_6H_5$ and $—CH_3$. Unless the substituent is halogen (amines 19, 20) it would seem that both positions *ortho* to the amino group should be unoccupied (amines 22, 25, 26). This may explain the apparent anomalies of negative reactions with the naphthalene analogues of sulphanilic acid and its amide (amines 11, 12). With the amino group in the 2-position in naphthalene (amine 13) the reaction was strongly positive.

Derivatives of aniline substituted in the *para* position by $—NO_2$, $—COCH_3$, $—COOH$, $—COOC_2H_5$, $—SO_3H$, $—SO_2NH_2$, $—SO_2NHR$, and $—SO_2NRR'$ all gave a positive reaction although not all of the pigments formed were of equal intensity of colour, and they varied in their degree of preference for the aqueous or *n*-butanol phase. The group of sulpha drugs and 2:4-dichloroaniline gave the best results. N^1-Diethylsulphanilamide was made the subject of further study owing to the stability of its diazonium salt, the readiness with which the pigment transferred to the *n*-butanol phase and the light colour of the control. This amine was also more easily diazotized than 2:4-dichloroaniline and had the further advantage of freedom from halogen. There is, of course, the possibility that other amines may prove even more suitable, particularly since, in our subsequent studies with N^1-diethylsulphanilamide, the conditions for diazotization and colour development eventually adopted differed materially from those used in the above comparative tests.

Observations on the conditions of Komant's test

Komant described two sets of conditions leading to the formation of coloured products between thyroxine and diazotized sulphanilic acid: (*a*) in which equal volumes (1 ml.) of solutions of thyroxine (0·1 %), the diazonium chloride and Na_2CO_3 (10 %) were mixed to give a red colour (the colour tone changing gradually to brown although in more dilute solutions of thyroxine the colour was found to fade in a few minutes), and (*b*) in which 5 vol. of the diazonium chloride solution were added. Under the latter conditions Komant observed the production of a violet colour which rapidly faded on passing a current of oxygen through the solution. Qualitative observations have shown that these subsequent changes of colour are largely dependent upon pH. Thus, if under conditions (*a*) (pH 9–10) 2N-NaOH (1 ml.) is added immediately after colour development, the initial colour quality is retained for several days, even with dilute (0·005 %) solutions of thyroxine, while that of the control (no thyroxine) is straw rather than dark yellow. Similarly, under conditions (*b*) (pH about 8·5) the initial red-purple colour does not change to violet and then to brown, but persists for several days if alkali (1 ml. 10N-NaOH) is immediately added. Moreover, if NaOH is not added, the subsequent decompositions appear to be independent of reactions with oxygen, since in controlled tests the bubbling of oxygen, air or nitrogen effected the changes (without, however, the development of an intermediate violet coloration) at similar rates; even mechanical agitation of the solution in a closed tube caused the initial red-purple colour to pass rapidly to reddish orange, whereas that of the contents of a similar stationary tube changed gradually *through violet* to brown. The colour changes are probably mainly due to side reactions of diazotized sulphanilic acid, these being inhibited at high pH.

Investigations with N^1-diethylsulphanilamide. Effects of pH *and other factors on the production, stability and specificity of the colour*

Reaction at pH 11–12. Under the conditions used for the comparative test (pH 9–10, Table 1) diazotized N^1-diethylsulphanilamide also produced a colour with diiodotyrosine (orange red) and tyrosine (deep yellow) while the control was yellow. Although diiodotyrosine can be separated from thyroxine by partitioning between aqueous NaOH and *n*-butanol, tyrosine is more evenly distributed between the two phases. A differentiation was made possible by carrying out the reaction at a higher pH as described below. It may be noted that colour development also depends upon the order of mixing of the reactant solutions, thyroxine, diazonium salt and alkali and *n*-butanol and that the addition of a further amount of alkali both intensifies and stabilizes the colour initially produced (as mentioned above for the colour formed with diazotized sulphanilic acid).

Thyroxine solution (0·004 %; 1 ml.) was mixed with *n*-butanol (1 ml.) and then 1 ml. of aqueous NaOH of selected concentration was run in. The mixture was shaken and the solution of the diazonium chloride (1 ml.) was then added and shaking continued for 30 sec. 2N-NaOH (1 ml.) was then immediately added. The results of these tests and of those carried out at the same time with diiodothyronine, diiodotyrosine, tyrosine and control solutions are given in Table 2. It can be seen that the range 0·2–0·3N-NaOH provides optimum conditions (pH 11–12) for the production of an immediate colour of good intensity with a pale control and negative results with tyrosine and diiodotyrosine. The alkali initially added, if below 0·15N, produced only yellow or brown colours even after 24 hr., whereas if above 0·5N, a colour developed only on standing. In order to check the optimum pH for the development of an immediate colour, a series of tests was carried out with buffer solutions over the pH range 10–12·8. The buffer was formed from glycine, NaOH and NaCl (Walbaum, 1920). The solution of thyroxine (0·1 %; 1 ml.) in buffer at the test pH was shaken with butanol (1 ml.), and 1 ml. of the diazo solution (previously brought to pH 7) was added. The results confirmed the pH range 11–12 as optimal; the conditions of the buffer medium were not, however, suitable for general adoption, as the control was yellow (due to glycine) with a corresponding yellow tint in the test colour.

Table 2. *Colours developed between diazotized N^1-diethylsulphanilamide and thyroxine and related compounds at varying* pH (*strength of alkali added as indicated*). *Reaction in water—*n*-butanol. 30 sec. after mixing the reagents additional alkali* (2N-NaOH; 1 *ml.*) *was added to the tubes and the colours of the butanol phases noted; after standing for* 24 *hr. the colours were again observed*

NaOH (1 ml.) ...	0·05N		0·1N		0·2N		0·3N		0·5N		N	
Time ...	30 sec.	24 hr.	30 sec.	24 hr.	30 sec.	24 hr.	30 sec.	24 hr.	30 sec.	24 hr.	30 sec.	24 hr.
Thyroxine (0·004 %; 1 ml.)	Yellow	Orange	Brown	Brown	Light purple	Light purple	Deep pink	Light purple	Pale pink	Deep pink	No colour	Pink
3:5-Diiodothyronine (0·004 %; 1 ml.)	Yellow	Brown	Brownish pink	Orange brown	Pink	Pink	Pale pink	Light pink	Straw	Pale pink	No colour	Straw
3:5-Diiodotyrosine (0·004 %; 1 ml.)	Yellow	Yellow	Deep yellow	Yellow	Straw	Light yellow	No colour	Straw	No colour	Straw	No colour	Straw
Tyrosine (0·004 %; 1 ml.)	Light yellow	Yellow	Deep yellow	Deep yellow	No colour	Straw	No colour	Straw	No colour	Straw	No colour	Straw
Control (no amino-acid)	Yellow	Yellow	Yellow	Yellow	No colour	Straw	No colour	Straw	No colour	Straw	No colour	Straw

Reaction in butanol phase with excess alkali. Although the depth of colour even in dilute solutions was quite satisfactory when the reaction was carried out at pH 11–12 followed by the addition of 2 N-NaOH, duplicate values did not show sufficiently close agreement when the reaction was examined quantitatively in the Spekker Absorptiometer (Adam Hilger) under these conditions. After various additional trial experiments it was found that replication of tests could be achieved by allowing the reaction to proceed slowly in the butanol phase, excess alkali being added initially. Under the conditions described below in *Procedure*, it was found by titration that about 5% of the excess alkali in the reaction mixture was in the butanol phase. Since thyroxine is completely transferred to the butanol layer in aqueous alkali-butanol mixtures, this procedure also permitted a maximum reaction to take place while enhancing the specificity. The solution of the diazo compound as well as that of the dye possesses marked stability at high pH. After the addition of the alkaline diazo solution, the reaction can be interrupted at any stage by simply stoppering the tubes, and then started again by allowing access to the atmosphere. Neither light nor temperature has a deteriorating effect upon the reaction provided the pH is maintained at a high level. Thus, after the diazonium hydroxide was added in one test, the tubes were placed in a boiling water bath for 0·5 hr., but neither the control nor thyroxine tubes showed any apparent change. However, on standing overnight at room temperature, the normal reaction took place in the thyroxine tube. On the other hand, elevating the temperature during the reaction by placing the tubes in a thermostat at 25° reduced the time for full colour development. Continual gentle agitation, while not essential, was found to assist the reaction by equalizing the fall in pH in the butanol layer. Violent shaking, on the other hand, facilitated diffusion of alkali from the aqueous phase, so that the pH did not fall to a level for colour development.

The mechanism of the reaction probably depends on the *slow* absorption of CO$_2$ from the atmosphere by the NaOH contained in the butanol phase, as no colour develops when the tubes are stoppered, or when CO$_2$-free air is passed over the butanol surface. Various attempts were made to speed up the reaction by the addition of minimal amounts of dilute acid, buffer solutions or salts, or by passing CO$_2$ over the butanol surface; however, neither duplicates nor colour quality were as satisfactory as by the slow process. The reaction was also tried unsuccessfully in *n*-butanol with all solutions prepared in this solvent, the diazotization being carried out with *n*-butyl nitrite.

Specificity. Table 3 gives a list of phenols, diiodophenols and amino-acids tested under the procedure ultimately adopted and described below. Thyroxine and thyroxamine produce about the same depth of colour, while that from diiodothyronine is very much weaker. Thyronine behaves like diiodotyrosine and tyrosine. The peptide, glutamylthyroxine, also gives a negative reaction under these conditions; on the other hand, thyroxyldiiodotyrosine, owing to its preference for the butanol phase, gives a stronger reaction, at equivalent dilution, than thyroxine.

Table 3. *Reactions between diazotized N¹-diethylsulphanilamide and various hydroxy compounds and amino-acids, compared with thyroxine*

Compound	Conc. (%)	Colour produced: Immediately	Colour produced: After 24 hr.
Thyroxine	0·005	No colour	Light purple
Thyroxamine	0·005	No colour	Light purple
3:5-Diiodothyronine	0·005	No colour	Pale pink
Thyronine	0·005	No colour	Straw
3:5-Diiodotyrosine	0·02	No colour	Straw
Tyrosine	0·02	No colour	Straw
Control	—	No colour	Straw
Glutamylthyroxine	0·005	No colour	Very light yellow
Thyroxyldiiodotyrosine	0·005	No colour	Purple
3:5-Diiodotyrosine methyl ester	0·005	No colour	Straw
3:5-Diiodo-4-hydroxybenzoic acid	0·005	Straw	Yellow
3:5-Diiodo-4-hydroxybenzaldehyde	0·005	No colour	Straw
*3:5-Diiodo-4-hydroxyphenyllactic acid	0·02	No colour	Yellow
3:5-Diiodo-*p*-cresol	0·005	Pink	Deep red
p-Cresol	0·02	No colour	Deep pink
p-Hydroxyphenyllactic acid	0·02	No colour	Yellow
p-Hydroxyphenylpyruvic acid	0·02	No colour	Yellow
p-Hydroxybenzoic acid	0·02	No colour	Yellow
Salicylic acid	0·02	No colour	Yellow
D-Glutamic acid	0·02	No colour	Light yellow
DL-β-Phenylalanine	0·02	No colour	Light yellow
L-Histidine	0·02	Yellow	Deep yellow
L-Hydroxyproline	0·02	No colour	Light yellow
L-Tryptophan	0·02	No colour	Yellow
Glycine	0·02	No colour	Light yellow

* Saul & Trikojus (1948) reported the value of diazotized N¹-diethylsulphanilamide in enabling them to follow the course of the conversion of 3:5-diiodo-4-hydroxyphenyllactic acid into the corresponding analogue of thyroxine.

Both components of this dipeptide presumably engage in reaction with the diazo compound. Of the monocyclic types tested, only *p*-cresol and its diiodo derivative gave a positive result.

It should be pointed out that some of the substances tested have not been shown to occur naturally; others could be removed by procedures which would not affect thyroxine. Thus *p*-cresol and its diiodo derivative are readily soluble in ether, and mixtures of these phenols and thyroxine have been shown after ether treatment to match the colour given by the same amount of thyroxine treated similarly. Complete hydrolysis should eliminate irregularities due to contamination by a peptide such as thyroxyldiiodotyrosine. This would also be necessary in a method depending upon the estimation of the iodine content of a butanolic extract.

METHOD

Reagents

Preparation of N^1-diethylsulphanilamide. 4-Acetamidobenzenesulphonyl chloride (40 g.) was dissolved in acetone (200 ml.) and added to a cold aqueous solution of diethylamine, prepared from diethylamine hydrochloride (44 g.) and 2 N-NaOH (200 ml.). The mixture was chilled, powdered $NaHCO_3$ (16 g.) added and the reaction allowed to proceed at room temperature for some hours with shaking at frequent intervals. Acetone was removed on the water bath under reduced pressure, the residue was chilled and water (200 ml.) added. The precipitate of N^1-diethyl-4-acetamidobenzenesulphonamide which separated was hydrolyzed by boiling for a few minutes with 6 N-HCl (40 ml.). Following the addition of water, heating was continued for a further few minutes. The hot filtered solution was cooled and made alkaline with 2·5 N-NaOH. The resulting precipitate was washed with water (crude yield, 32 g.) and recrystallized, first, from 60% (v/v) ethanol with the aid of charcoal, and then from a mixture of ethanol and light petroleum (b.p. 60–80°). It separated as colourless prisms, m.p. 105–106°. (Mangini (1940) records 105–106°.) Found: N, 12·5%. (Calc. for $C_{10}H_{16}O_2N_2S$: N, 12·3%.)

Diazotization. Preliminary tests showed that the large excess of $NaNO_2$ used by Komant was not necessary for the production of a stable solution of the diazonium salt of this amine. Accordingly, the diazotization was carried out as follows: the amine (1·14 g., 0·005 mol.) was dissolved in 10 N-HCl (9 ml.) and the solution diluted to 100 ml. with distilled water. 5 ml. of this solution was diazotized at 0° with 5 ml. of 4·5% (w/v) $NaNO_2$ (A.R.); ice-cold water (20 ml.) was then added and the resulting solution used after 10 min. Reduction of the quantity of nitrite did not affect the rate of colour development with thyroxine, while the control was somewhat paler.

n-*Butanol.* This was purified by distillation over solid NaOH; the fraction boiling at 116° was used in this work.

Procedure

The procedure ultimately used for quantitative tests was as follows: thyroxine (2 ml. of a solution containing 1–5 mg./100 ml. of 0·1 N-NaOH) was placed in a glass-stoppered round-bottomed Pyrex centrifuge tube of approx. 15 ml. capacity, followed by *n*-butanol (2 ml.) and then 2 N-NaOH (1 ml.). The contents were then thoroughly mixed by shaking the tube sideways (liquid should not pass to the neck of the tube). An alkaline solution of the diazo derivative of N^1-diethylsulphanilamide (3 ml., prepared by adding 1 ml. of 2 N-NaOH to 2 ml. of the solution of the diazonium chloride was then added, the tube stoppered and the contents well shaken for 15 sec. After standing for 5 min. the stopper was removed and the tube gently agitated in a rack for 24 hr. in a water thermostat maintained at 25°. The rack, the base of which was suspended at a distance of 17 cm., was swung by a geared electric motor about 60 times/min. through approx. 3 cm. (the dimensions given are not critical). The control tube contained 2 ml. of 0·1 N-NaOH plus reagents. After the completion of the reaction, the tubes were stoppered and centrifuged for 30 min. at 2500 r.p.m. The butanol phase was then transferred as far as possible with a Pasteur pipette to a small dry tube. To 0·75 ml. of this solution 0·25 ml. N-ethanolic NaOH was then added and the tubes containing the mixed solutions stoppered pending examination in the absorptiometer using Ilford spectrum green filters. Tests showed that almost identical readings could be obtained after a further 24 hr., providing precautions were taken to prevent evaporation of solvent and access of CO_2. (Note. If ethanolic NaOH is not added, the readings are irregular due to the development of a turbidity. Other measures to counteract this, such as more prolonged centrifuging, temperature stabilization or desiccation with anhydrous Na_2SO_4, were ineffective.)

Absorption characteristics. Fig. 1 shows the result of an investigation of the spectral transmittance of the solution of the dye in *n*-butanol (plus ethanolic NaOH). Comparison was with a control solution prepared under identical conditions, thyroxine only being excluded. The instrument used, the Coleman Spectrophotometer, was kindly made available by Mr A. T. Dick (Animal Health Laboratory, Council for Scientific and Industrial Research, Melbourne). Maximum absorption occurred at approx. 540 mμ. at which point the blank control transmitted 93·6% compared with pure *n*-butanol.

Standard curve. A typical calibration curve of the colour intensity produced by varying concentrations of pure thyroxine is illustrated in Fig. 2. Within the range 1–4 mg./100 ml. consistent results have been obtained provided fresh solutions of thyroxine were used for the standardization. In applying the method to the estimation of thyroxine in biological material, such as thyroid powder, some difficulty was experienced in obtaining suitable butanol extracts of the thyroxine fraction prior to assay. This has now been overcome by preliminary treatment with ether before extracting the acid-insoluble thyroxine precipitate with butanol or by forcing the thyroxine into an aqueous alkaline phase from a butanol extract by the addition of ether or, preferably, light petroleum. Re-extraction of the aqueous phase with butanol has provided a colourless solution suitable for colorimetric analysis. A report of the results obtained in these studies will form the basis of a further communication.

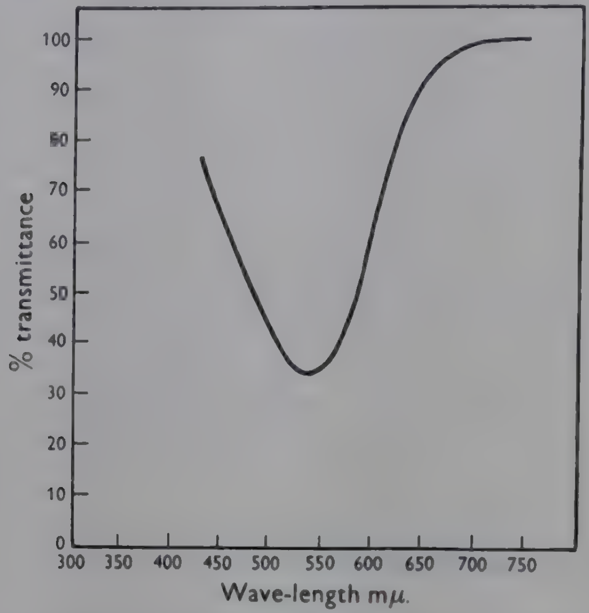

Fig. 1. Spectral transmittance of the colour produced by the reaction of thyroxine (0·01 %) and diazotized N^1-diethylsulphanilamide. Coleman spectrophotometer.

Note added 10 *October* 1947.

Since this paper was submitted for publication we have had access to a preliminary report by Moser (1947), in which he has shown that by the addition of alkali to the reaction product, Komant's reagent may be used for the rapid estimation of thyroxine in pure aqueous solutions. Our observations indicate that N^1-diethylsulphanilamide is a more stable reagent for this purpose and that the procedure outlined by us greatly enhances the specificity of the reaction.

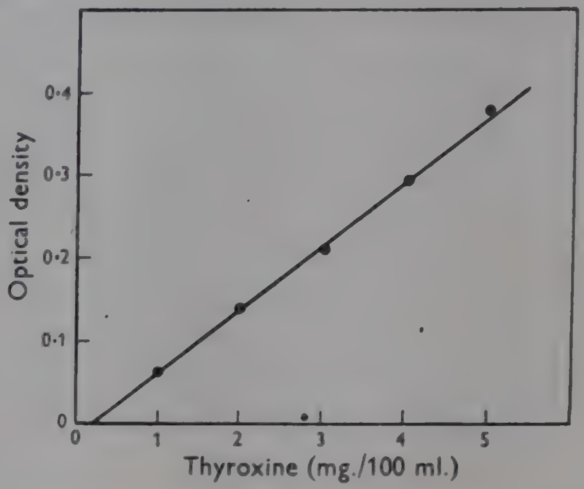

Fig. 2. Calibration curve for the reaction: thyroxine + diazotized N^1-diethylsulphanilamide. Hilger Spekker Absorptiometer with Ilford spectrum green filters.

SUMMARY

A colour test for thyroxine is described based upon its reaction with diazotized N^1-diethylsulphanilamide in alkaline solution. The conditions of the reaction have been studied and a procedure has been suggested whereby thyroxine can be colorimetrically estimated.

The Australian National Health and Medical Research Council made a personal grant to one of us (D. W.) and also contributed to the expenses of the investigation. We are indebted to Dr C. R. Harington, F.R.S., Director, National Institute for Medical Research, London, for the two specimens of thyroxine dipeptides.

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The Reactivity of the Combined Cystine of Proteins other than Wool

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Previous work has shown that the action of bisulphite solutions on wool leads to the subdivision of the combined cystine into two main fractions of differing chemical reactivity (Elsworth & Phillips, 1941; Middlebrook & Phillips, 1942*a*), each of which can be divided into two fractions showing smaller differences in reactivity. Further work on the reaction of wool with formaldehyde (Middlebrook & Phillips, 1942*b*, 1947) and alkalis (Cuthbertson & Phillips, 1945; Lindley & Phillips, 1945) has provided evidence that the combined cystine can be subdivided by reagents other than bisulphite. The effect of chemical modification of the wool on the magnitudes of the cystine fractions has been studied (Lindley & Phillips, 1947) and has helped to interpret the results in terms of protein structure.

In these circumstances it appeared important to determine whether the reactivity of the combined cystine varies in all cystine-containing proteins, or whether such variation is confined to the keratins or even to wool itself. The S distributions of a number of different types of keratin and of some other nonfibrous proteins have therefore been determined. The results obtained show that this variation in reactivity of the combined cystine is characteristic of the keratins and, with some slight reservations, of other cystine-containing proteins. The combined cystine of all the keratins examined has thus been found to be divisible into two main fractions: $(A+B)$ defined as combined cystine which yields thiol plus S-cysteinyl sulphonate groups with $NaHSO_3$ and lanthionine with dilute alkalis, and $(C+D)$ defined as being inert towards cold $NaHSO_3$ and giving combined α-aminoacrylic acid with alkalis. Fraction $(A+B)$ of these proteins has been further divided into subfraction A which gives water-unstable thiol and S-cysteinyl sulphonate groups with $NaHSO_3$, and subfraction B, which gives water-stable thiol and S-cysteinyl sulphonate groups. No attempt has been made to differentiate between subfractions A and B of the non-keratinous proteins.

THE $(A+B)$ AND $(C+D)$ FRACTIONS OF THE COMBINED CYSTINE OF VARIOUS KERATINS

EXPERIMENTAL

Materials. These call for no special comment beyond the fact that they were purified before analysis by successive extraction with ethanol and water, the sole exceptions to this being the cow- and sheep-horn samples which are asterisked in Tables 1–4. After these latter had been partially analyzed, it was discovered that they had previously been boiled in water for some hours to remove the non-keratinous cores. New material was accordingly analyzed, but both sets of analyses are recorded for completeness. To facilitate penetration of the $NaHSO_3$ solution, all the horn samples were converted to fine shavings in a milling machine.

Analytical methods. These are identical with those previously described (Lindley & Phillips, 1945, 1947) with the addition of amide-N estimations, which were carried out as follows: 5 ml. samples of the hydrolysates (1 g. hydrolyzed for 4 hr. with 10 ml. 5N-HCl, filtered and made up to 50 ml.) were transferred to micro-Kjeldahl flasks and made just alkaline (bromocresol green) with 2N-NaOH; 5 ml. of 2% (w/v) borax were added to bring the pH to 8–9, and the NH_3 removed by distillation in a micro-Kjeldahl apparatus.

RESULTS

The S *distribution of the keratins*

The results of various S estimations on a variety of keratins are given in Table 1 together with the percentage of S unaccounted for either as cystine, cysteine, methionine, Br-oxidizable S or sulphate S. It will be seen that the keratins have a wide range of values for cysteine, cystine, total and unidentified S. Particularly noteworthy are the high cystine content of the dog hair and the high cysteine content of the two horn samples first analyzed. This high thiol content could not be duplicated on the other two samples by boiling them with water, and therefore this probably represents a real difference between the two specimens. The wide variation in the analytical figures of the various horn samples is of some interest.

Another point of importance is the wide range of values found for the unidentified S of the keratins. Thus the total S of dog hair, sheep horn and duck feathers can almost quantitatively be accounted for as cystine, methionine and Br-oxidizable + sulphate S, whereas calf, cow-tail and rabbit hair all contain approximately 0·8 % S which remains unidentified. That this is not necessarily due to treatment of the keratin prior to analysis is established conclusively in the case of the rabbit hair. This particular specimen was obtained from the rabbit used to prepare the myosin for the later experiments and had no subsequent treatment other than extraction with ethanol and water. It also seems improbable that this unidentified S arises by oxidation of cystine

Table 1. *The S distribution of certain keratins*

(Results as % of protein dry wt.)

Keratin	Total S	Sulphate S + Br-oxidizable S	Thiol S	Disulphide S	Methionine S	Unidentified S (by diff.)
Dog hair	5·30	0·29	0·15	4·65	0·16	0·05
Rabbit hair	4·35	0·27	0·16	2·94	0·22	0·76
Calf hair	3·97	0·45	0·05	2·53	0·07	0·87
Cow-tail hair	3·93	0·28	0·05	2·71	0·10	0·79
Horse hair	3·71	0·19	0·00	2·83	0·19	0·50
Camel hair	3·28	0·09	0·00	2·74	—	—
Alpaca	3·93	0·14	0·00	3·34	0·08	0·38
Yak hair	3·85	—	0·00	2·78	—	—
Reindeer hair	—	—	0·00	2·40	—	—
Duck feathers	3·26	0·19	0·01	2·75	0·10	0·21
Cow horn*	4·17	0·37	0·54	2·37	0·08	0·81
Sheep horn*	—	0·19	0·34	2·68	—	—
Cow horn	3·77	0·07	0·22	2·94	0·14	0·40
Sheep horn	2·08	0·01	0·10	1·69	0·14	0·14
Foetal lamb's wool	3·64	0·08	0·13	2·95	—	—

* Pre-boiled in water.

Table 2. *The subdivision of the combined cystine of keratins into fractions $(A+B)$ and $(C+D)$*

(Results in terms of S as % of protein dry wt.)

Keratin	Fraction $(A+B)$	Fraction $(C+D)$ (by diff.)	Subfraction B	Subfraction A (by diff.)
Dog hair	2·52	2·28	0·64	1·88
Rabbit hair	1·63	1·47	0·40	1·23
Calf hair	1·13	1·45	0·35	1·10
Cow-tail hair	1·62	1·14	0·69	0·93
Horse hair	1·75	1·08	0·46	1·29
Camel hair	1·39	1·35	0·35	1·04
Alpaca	1·37	1·97	0·52	0·85
Yak hair	0·96	1·82	0·31	0·65
Reindeer hair	0·77	1·63	0·27	0·50
Duck feathers	2·04	0·72	0·86	1·18
Cow horn*	2·03	0·88	0·50	1·53
Sheep horn*	1·56	1·46	—	—
Cow horn	2·22	0·94	0·63	1·59
Sheep horn	1·20	0·59	0·34	0·86

* Pre-boiled in water.

during hydrolysis, since Baernstein estimations (in which the hydrolysis is carried out in a reducing medium throughout) gave values of 2·48, 2·72 and 3·16 % S respectively for the calf hair, cow-tail hair and rabbit wool compared with 2·58, 2·76 and 3·10 % S for the thiol + disulphide S by Shinohara estimation. Two-dimensional partition chromatography on filter paper of the hydrolysate of rabbit wool did not reveal the presence of lanthionine or any new amino-acid. A very faint spot corresponding to cysteic acid did occur, but was not of sufficient intensity to account for the unidentified S. Pending the result of further investigations, now in progress, the question of the chemical nature of this unidentified S must therefore be left open.

The subdivision of the combined cystine into subfractions A and B and fraction (C + D)

The various keratins were treated with 30 % (w/v) $NaHSO_3$ overnight, hydrolyzed and the thiol S content of the hydrolysates estimated to give fraction $(A+B)$. Fraction $(C+D)$ was calculated by subtracting this value from the disulphide S (Table 1). The results obtained are given in Table 2, together with the determinations of subfraction B and calculated values for subfraction A.

From Table 2 it will be seen that all the proteins contain both fractions $(A+B)$ and $(C+D)$ and that the magnitude of these fractions varies both absolutely and relatively. When the original fractionation of the cystine S of wool was first reported (Middlebrook & Phillips, 1942*a*) some stress was laid on the approximate equality of fractions $(A+B)$ and $(C+D)$ of wool. The present analyses show that this is not a general property of keratins. By virtue of its very high cystine content, dog hair has the highest absolute value for both fractions $(A+B)$ and fraction $(C+D)$. The next highest absolute values for fraction $(A+B)$ are shown by duck feathers and cow horn, but if fraction $(A+B)$ is expressed as a percentage of either the total S or the total disulphide S, then duck feathers easily provide the highest value. This is of some interest since duck feathers are the only representative of the naturally occurring β-keratins here analyzed. The keratin with the lowest $(A+B)$ fraction both absolutely and relatively was reindeer hair, which was also undoubtedly the least impressive fibre of the varieties studied, being of very irregular cross-section, and with little tensile strength.

Table 3. *Ratio between fraction $(A+B)$ and total and disulphide* S, *and between subfractions* A *and* B, *together with amide*-N *values*

Keratin	$(A+B)$/Total S	$(A+B)$/Disulphide S	A/B	Amide N (% of dry wt.)
Dog hair	0·48	0·53	2·9	1·11
Rabbit wool	0·37	0·53	3·1	1·07
Calf hair	0·28	0·44	3·1	1·20
Cow-tail hair	0·31	0·59	1·35	1·26
Horse hair	0·41	0·62	2·8	1·22
Camel hair	0·42	0·51	3·0	1·25
Alpaca	0·35	0·41	1·6	1·27
Yak hair	0·25	0·35	2·1	1·34
Reindeer hair	—	0·32	1·9	1·39
Duck feathers	0·62	0·74	1·4	1·31
Cow horn*	0·49	0·70	3·1	1·24
Sheep horn*	—	0·52	—	—
Cow horn	0·59	0·70	2·5	1·14
Sheep horn	0·58	0·67	2·5	1·20

* Pre-boiled in water.

Whilst fraction $(A+B)$ does not in general form any simple fraction of either the total disulphide S or total S a rather remarkable regularity comes to light when the relative magnitudes of subfractions *A* and *B* are compared (Table 3). In six of the keratins, dog hair, cow horn, rabbit wool, calf hair, horse hair and camel hair, subfraction *A* is approximately three times as great as subfraction *B*. In two of the keratins, yak hair and reindeer hair, the ratio is 2 to 1, whilst in cow-tail hair and duck feathers it is approximately 4 to 3; the alpaca gives an isolated figure of 1·6 (3) to 1, and the cow and sheep horn each give a ratio of 2·5 to 1. The ratio of subfraction *A* to subfraction *B* in the wool analyzed by Lindley & Phillips (1947) was 2 to 1.

Lindley & Phillips (1947) pointed out that subfraction *A* of the combined cystine of wool appeared to be intimately associated with the free carboxyl groups, since esterification of these carboxyl groups altered the cystine reactivity so that this subfraction behaved like subfraction *B*. A not unreasonable assumption, therefore, is that subfraction *B* is associated with amide groups of the wool. Accordingly the amide-N contents of the keratins were determined, Table 3, where it is seen that no obvious correlation exists between the magnitude of subfraction *B* of the various keratins and their amide-N contents.

THE $(A+B)$ AND $(C+D)$ FRACTIONS OF THE COMBINED CYSTINE OF SOME NON-KERATINOUS PROTEINS

Experimental

Materials. (1) Egg albumin: A commercial sample from British Drug Houses Ltd. and also a crystalline preparation made by the procedure of Kekwick & Cannan (1936). (2) Gliadin: This and the two succeeding proteins were gifts from Prof. A. C. Chibnall. (3) Edestin: Recrystallized. (4) Ground nut globulin. (5) Rabbit myosin: Prepared from the leg muscle of the rabbit according to the method of Greenstein & Edsall (1940). (6) Insulin: British Drug Houses Ltd., crystalline. (7) Lactoglobulin: This was prepared according to Palmer (1934), but could not be induced to crystallize and was eventually precipitated from solution by the addition of ethanol. (8) Serum albumin: A crude commercial preparation (British Drug Houses Ltd.).

Treatment with NaHSO$_3$. The protein (*c.* 0·5 g.) was treated with NaHSO$_3$ (1 g.) and water (3 ml.) overnight. Conc. HCl (5 ml.) was then added and the mixture boiled under reflux for 4 hr. Control experiments have shown that this method can be used to determine fraction $(A+B)$ satisfactorily for wool.

Results

Tables 4 and 5 give the results of the analyses together with those previously reported by other investigators. The results were calculated on a dry weight basis calculated from the N content (Kjeldahl) of the hydrolysates. From these results it is apparent

Table 4. *The thiol and disulphide* S *content of protein hydrolysates*

(Results in terms of S as % of protein dry wt.)

Protein*	Experimental results			Other investigators			Reference
	SH	SS	SH + SS	SH	SS	SH + SS	
Egg albumin (commercial) (15·8% N)	0·07	0·45	0·52	—	—	—	—
Egg albumin (crystalline) (15·8% N)	—	—	—	0·37	0·28	0·65	Hess & Sullivan (1943)
	0·17	0·12	0·29	—	—	0·48	Kassell & Brand (1938)
Myosin (16·8% N)	0·0	0·19	0·19	0·29	0·04	0·33	Hess & Sullivan (1943)
	—	—	—	—	—	0·21	Bailey (1937 *a*)
Gliadin (17·4% N)	0·01	0·48	0·49	—	—	0·64, 0·69	Bailey (1937 *b*)
Edestin (18·6% N)	0·0	0·32	0·32	0·09	0·26	0·35	Hess & Sullivan (1943)
	—	—	—	—	—	0·38	Lugg (1938)
Lactoglobulin (15·6% N)	0·11	0·71	0·82	0·29	0·61	0·90	Brand & Kassell (1942)
Insulin (15·5% N)	0·0	2·60	2·60	0·0	3·32	3·32	Miller & du Vigneaud (1937)
Insulin HCl-HCOOH	0·0	2·96	2·96	—	—	—	—
Serum albumin (15·7% N)	0·0	1·25	1·25	0·08	1·57	1·65	Hess & Sullivan (1943)
Ground nut globulin (18·3% N)	0·02	0·20	0·22	—	—	—	—

* The N content used in the calculation of protein wt. is given in parenthesis.

Table 5. *Fraction* $(A+B)$ *of the combined cystine of various proteins*

(Results in terms of S as % of protein dry wt.)

Protein	SH of fraction $(A+B)$	SS + SH	Maximum SH in protein	Reference
Egg albumin (commercial)	0·30	0·53	—	—
Egg albumin (crystalline)	0·15	0·36	0·34, 0·26	Greenstein (1938), Mirsky (1941)
Myosin	0·08	0·28	0·31	Greenstein & Edsall (1940)
Gliadin	0·26	0·56	—	—
Edestin	0·18	0·31	0·13	Greenstein (1939)
Lactoglobulin	0·38	0·81	—	—
Insulin	0·72	2·56	0·0	Greenstein (1944)
Serum albumin	0·70	1·08	0·09	Greenstein (1940)
Ground nut globulin	0·06	0·23	—	—

that the combined cystine of non-keratinous proteins can also be divided into fractions which are reactive and unreactive to $NaHSO_3$ solutions at pH 5 and room temperature. In most of these cases the $(A+B)$ fraction is approximately half of the total disulphide, the principal exceptions being myosin and insulin, and with both these complications result from losses of S during hydrolysis. The confirmation of the finding of Miller & du Vigneaud (1937) that increased recovery of cystine from insulin can be obtained by hydrolyzing in the presence of formic acid is of interest since unpublished work by Mr W. R. Middlebrook in this laboratory has shown that this technique of hydrolysis does not affect the recovery of cystine from wool. Although the present value for the cystine content of insulin is lower than that of Miller & du Vigneaud it is in agreement with the work of Sahyun (1941) who prepared crystalline insulin with 2·96% total S. The present value has also been checked by Baernstein's procedure which gave 2·90% S as cystine + cysteine.

DISCUSSION

It has long been realized that the sulphur and cystine contents of keratins vary widely and that this variation occurs even within one type of keratin, e.g. wool. It seemed possible, however, that some regularities might be revealed by considering the differing cystine fractions separately, but the results obtained in the present work lend little support to this view. A possible exception to this is that subfraction *A* may be a simple multiple of subfraction *B*, but even this doubtful relationship is not constant throughout the keratins.

The observation that the combined cystine of the other proteins examined also shows differing reactivity towards $NaHSO_3$ solutions is important, since it suggests that the ultimate cause of this effect will be found in some relatively simple factor of protein structure. These results also make it extremely improbable that the differing reactivity of the various fractions of the combined cystine of wool are directly due to the heterogeneous nature of wool. It was also considered possible that the $(A+B)$ fraction of the non-keratinous proteins might be identical with the maximum titratable thiol groups found for these proteins in solution, but it cannot be claimed that the results lend much support to this suggestion. On this point the results are in conflict with those of Hess & Sullivan (1943) who found the cysteine content of the hydrolysates of a number of proteins to be equivalent to the titratable thiol groups of the intact protein. Unfortunately few independent estimations are available, but it would seem that in the case of myosin the present results are in agreement with the findings of Bailey (1937 *a*, *b*).

SUMMARY

1. It has been shown that the combined cystine of a number of keratins can be subdivided into fractions of differing reactivity towards $NaHSO_3$ solutions in a manner analogous to that previously found for the combined cystine of wool.

2. Evidence of a similar subdivision of the combined cystine S has been found for other non-fibrous proteins.

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Preparation and Antibacterial Action of some Compounds Structurally Related to Glutamic Acid. Their Application in Microbiological Determination of Small Quantities of Glutamine

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In studying the metabolism of glutamine (McIlwain, Roper & Hughes, 1948) use was made of compounds structurally related to glutamic acid which differentially inhibited certain of the reactions concerned. These inhibitors were selected from a larger group of analogues and derivatives of glutamic acid, whose preparation and properties are described in the present paper.

The substances concerned are listed in Table 1. Expectation that γ-glutamylhydrazine might be inhibitory was based on analogous findings with respect to pantoylhydrazine (Madinaveitia, Martin, Rose & Swain, 1945; McIlwain & Hughes, 1945). Inhibition by α-amino-γ-methylsulphinylbutyric acid or 'methionine sulphoxide' was reported while the present investigations were in progress (Borek, Miller, Scheiness & Waelsch, 1946; Waelsch, Owades, Miller & Borek, 1946). Several examples are known of inhibition by homologues of biologically active compounds and this led to the examination of the α-amino dicarboxylic acids of Table 1.

Table 1. *Effects on streptococcal growth, glycolysis and glutamine hydrolysis, of compounds structurally related to glutamine*

((*a*) The growth response of *Streptococcus faecalis* (laboratory strain, *F*) was examined in the medium employed in assay, but lacking the yeast preparation and methionine sulphoxide; for the Richards strain (*R*), glutamine (final concentration, 5×10^{-5} M) was added. Substances were examined at the following concentrations: 2×10^{-2} M, 4×10^{-3} M and 8×10^{-4} M, except in the cases of compounds (1), (3) and (6) when the highest concentration was 10^{-2} M. No compound replaced glutamine in permitting growth of the glutamine-requiring streptococci nor in stimulating streptococcal glycolysis. γ-Ethyl glutamate behaved towards the exacting streptococci in a fashion similar to glutamic acid, in that high concentrations permitted a delayed growth in absence of glutamine. This did not occur with the other compounds examined, and was presumably due to glutamic acid formed from the ester.

(*b*) Glycolysis was carried out anaerobically in $NaHCO_3$-NaCl solution (cf. McIlwain, 1946). Compounds were examined at the following concentrations: (2), (4), (5), (7), (9) and (10): 0·033, 0·0067, and in some cases 0·0013 M; of (1), (3), (6), (8) 0·016 and 0·0016 M.)

Compound	(*a*) Effect on growth		(*b*) Effect on glycolysis or NH_3 formation from glutamine	
	Inhibition (+) or absence of inhibition (0)	Organisms examined	Inhibition (+) or absence of inhibition (0) not tested (−)	Organisms examined
I. α-Amino dicarboxylic acids				
(1) α-Amino-α-propylsuccinic acid	0	*R*	0	*R*
(2) α-Amino-β-butylsuccinic acid	0	*R*	0	*R*
(3) α-Amino-β-octylsuccinic acid	0	*R*	0	*R*
(4) α-Aminoadipic acid	0	*R*	0	*R*
(5) α-Aminopimelic acid	0	*R*	0	*R*
(6) α-Aminoundecane-αλ-dicarboxylic acid	0	*R*	0	*R*
II. Other amino carboxylic acids				
(7) α-Amino-α-methylvaleric acid	0	*R*	0	*R*
(8) α-Amino-β-furylpropionic acid	0	*R*	0	*R*
III. Methionine derivatives				
(9) α-Amino-γ-methylsulphinylbutyric acid	0 +	*R* *F*	0	*R, F*
(10) α-Amino-γ-methylsulphonylbutyric acid	0	*R, F*	−	
IV. Glutamic acid derivatives				
(11) *N*-(γ-Glutamyl)hydroxylamine	+	*R, F*	−	−
(12) γ-Glutamylhydrazine	+	*R, F*	+	*R*
(13) Acetone derivative of (12)	+	*R, F*	−	−
(14) γ-Ethyl glutamate	0	*R, F*	−	−

Knowledge that one of the analogues, namely methionine sulphoxide, inhibited the synthesis of glutamine from glutamic acid suggested its application in the microbiological determination of glutamine. A method for determining 10–20 mμmol. of glutamine was required in studying the metabolism of the compound. The existing methods, either chemical or enzymic (cf. Archibald, 1945) depend finally on determination of NH_3 produced from glutamine under conditions more or less specific for glutamine. If the NH_3 was estimated by photometric measurement of the colour yielded with Nessler's reagent, quantities of about 500 mμmol. of NH_3 could be determined in this way. Such methods were, however, not necessarily sensitive to 500 mμmol. of glutamine. Thus it was necessary that any NH_3 initially present, or produced non-specifically under the conditions of hydrolysis of glutamine, should be removed or determined separately by blank estimations. In many cases we desired to determine glutamine in the presence of up to 100 times its (molar) quantity of ammonium glutamate. This demanded a very complete removal or accurate estimation of the NH_3, and in practice the determination of glutamine by the NH_3 formed from it did not give satisfactory results.

The possible use of microbiological methods for determining glutamine presents problems of a different type. The growth response of virulent group A β-haemolytic streptococci was applied in detecting and roughly estimating glutamine in natural materials in 1939 (McIlwain, Fildes, Gladstone & Knight, 1939). Since that time glutamine has been described as stimulating the growth of other micro-organisms (cf. Knight, 1945) and in some cases an organism's response to glutamine has been studied in detail as a possible basis for assay of glutamic acid or glutamine. In all such cases, difficulty has been encountered in discriminating between the effects of glutamic acid, ammonium salts, and glutamine. Response to glutamic acid has been found slower than response to glutamine with both streptococci (McIlwain *et al.* 1939) and *Lactobacillus arabinosus* (Lewis & Olcott, 1944; Lyman, Kuiken, Blotter & Hale, 1945; Hac, Snell & Williams, 1945); and equimolar concentrations of the substances may finally give the same response, or glutamine may give the greater response. Hac *et al.* (1945) applied the different chemical properties of glutamic acid and glutamine, together with their effects on the growth of *L. arabinosus*, to the determination of glutamine.

In the present study it was preferred to try to obtain conditions of growth which more sharply discriminated between glutamic acid and glutamine. This was especially necessary in determining glutamine in the presence of a large excess of glutamic acid. Conditions were therefore chosen in which ammonium and glutamic acid salts were throughout present in a deficient basal medium consisting of glucose-peptone with various addenda. Recently passaged β-haemolytic streptococci did not grow in this medium within 5 days or so unless glutamine was added. With excess glutamine, maximum growth was usually reached in 18–24 hr. Sub-optimal concentrations of glutamine resulted in less growth, but although the graded response of Fig. 1*A* was obtained, this was not a suitable basis for the assay of glutamine. The reason for this can be seen by considering the wide differences between the curves obtained from readings at different periods of growth. With quantities of glutamine between 5 and 25 mμmol., readings increase 2- to 3-fold between 18 and 25 hr.; at 5 or 10 mμmol. readings increase 2- to 6-fold between 25 and 41 hr. Any substance, therefore, which was present in a material being assayed for glutamine, and which accelerated or retarded growth of the streptococci during assay, would cause large variations in the organism's response to a given quantity of glutamine.

Growth of the type shown in Fig. 1*A* appeared likely to be due to synthesis of glutamine by the organisms from ammonium glutamate. We therefore tried to obtain a more stable response to glutamine by adding α-amino-γ-methylsulphinylbutyric acid to the deficient basal medium. The glutamic acid content of this medium was about 10^{-3} M, and it was anticipated from the results of McIlwain *et al.* (1948) that, with the specimen of sulphoxide employed (a mixture of isomerides), a concentration of about 2×10^{-2} M would be required. Trial showed that this concentration offered no advantage over one of $1{\cdot}1 \times 10^{-2}$ M, which was that adopted in the method as described below. Media containing the sulphoxide afforded a much more stable response to glutamine. This is illustrated in Fig. 1*B*, which shows the increase in readings between 16 and 24 hr. to be no more than 25 % of their values at the earlier time. Three strains of β-haemolytic streptococci gave results similar to that of Fig. 1*B*, and of them the Richards strain was adopted for use in assay, as its response to glutamine is known to be highly specific (McIlwain, 1939).

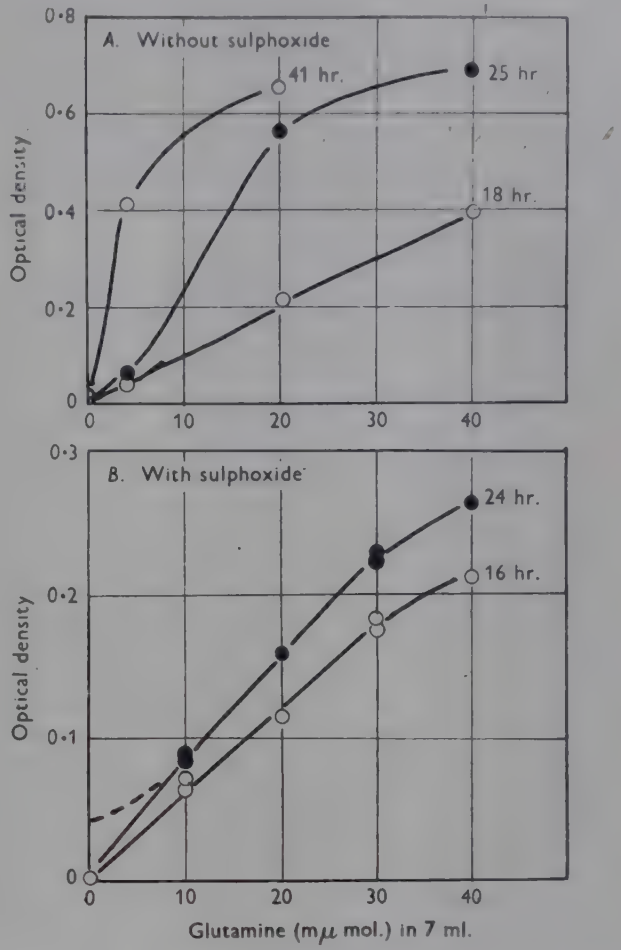

Fig. 1. Growth of streptococcal cultures in media initially containing varying quantities of glutamine, measured by their optical densities at different times. *B*, using the medium described in the text and normally employed in assay; *A*, using a medium without methionine sulphoxide, but otherwise identical with that of *B*.

EXPERIMENTAL AND RESULTS

Preparation of compounds related to glutamic acid

γ-Ethyl glutamate was prepared by the method of Bergmann & Zervas (1933) modified as follows. After esterification of glutamic acid (5 g.) in anhydrous ethanol (35 ml.) the volume was made up to 150 ml. with ethanol and British Drug Houses Ltd. Universal Indicator added. Aqueous NH_3 (sp.gr. 0·88) was added dropwise until the solution was orange-red, when the precipitated NH_4Cl was removed at the pump. The filtrate was cautiously made more alkaline with NH_3, any immediate amorphous precipitate being removed by filtration. The solution was finally brought to an orange-yellow colour, when the ester separated as plates. The whole was kept several hours at 0° and filtered, the filtrate being tested with NH_3 for a further precipitation of ester. After recrystallization from aqueous ethanol yields of more than 90 % were obtained.

γ-Glutamylhydrazine. γ-Ethyl glutamate (5 g.) with 50% (w/w) hydrazine hydrate (10 ml.) and water (5 ml.) were warmed at 70° for 15 min. Absolute ethanol (200 ml.) at 70° was added slowly and the mixture kept at 0° for several hours. The fine crystalline precipitate was filtered off and recrystallized from aqueous ethanol. A white crystalline solid (3·9 g., yield 72%; m.p. 165°) was obtained. (Melting points here and elsewhere are uncorrected.) (Found: C, 32·2, 31·5; H, 7·38, 7·42; N, 22·2%; loss in weight at 105°, 16·3%. $C_5H_{11}O_3N_3 . 1{\cdot}5H_2O$ requires C, 31·9; H, 7·44; N, 22·3; H_2O, 14·6%.) To minimize the possibility of glutamine production from γ-ethyl glutamate and NH_3 present as impurity in the hydrazine hydrate, the purest specimen of hydrazine hydrate available was used. This was estimated to contain 0·14 mol. % of NH_3.

Picrate. γ-Glutamylhydrazine (260 mg.) with picric acid (360 mg.) and water (30 ml.) was heated until complete solution was obtained. A yellow solid separated on cooling. This was filtered off and crystallized three times from water containing 5% (v/v) of ethanol. A fine yellow crystalline solid (445 mg., yield 78%; m.p. 190° (decomposition)) was obtained. (Found: C, 33·9; H, 3·48; N, 21·5%. $C_5H_{11}O_3N_3 . C_6H_3O_7N_3$ requires C, 33·9; H, 3·59; N, 21·5%.)

Acetone γ-glutamylhydrazone. γ-Glutamylhydrazine (0·29 g.) with acetone (2 ml.), ethanol (4 ml.) and water (1 ml.) was warmed on a water bath for 10 min. The clear solution obtained was kept at 0° for several hours. Acetone (50 ml.) was added. A microcrystalline solid separated and was filtered, washed with small portions of ice-cold ethanol and recrystallized from aqueous acetone, giving a white microcrystalline solid (150 mg.; yield, 70%; m.p. 171°). (Found: H, 7·58, 7·40, 7·65; N, 21·2%. $C_8H_{15}O_3N_3$ requires H, 7·46; N, 20·9%.)

Benzaldehyde γ-glutamylhydrazone. γ-Glutamylhydrazine (1 g.) with benzaldehyde (2 ml.) and ethanol (25 ml.) was heated on a water bath for 10 min. Water (25 ml.) was added gradually and a clear solution was obtained on continued heating. The solution was cooled slowly to 0° and held there for several hours. The white solid which slowly precipitated was filtered off and recrystallized from aqueous ethanol to give an odourless crystalline solid (1·06 g., yield 80%; m.p. 192° with decomposition). (Found: C, 57·5; H, 6·08%. $C_{12}H_{15}O_3N_3$ requires C, 57·8; H, 6·02%.)

β-Aspartylhydrazine. Asparagine monohydrate (10 g.) was refluxed with 50% (w/w) hydrazine hydrate (30 ml.) for 20 min. The solution was evaporated *in vacuo* to a gum which was taken up in water (15 ml.). Ethanol (70 ml.) was added gradually, the precipitated amorphous white solid filtered off, washed well with ethanol and crystallized from aqueous ethanol. It gave fine white crystals (9·0 g.; yield 92%; m.p. 194–195° with decomposition). (Found: C, 32·8; H, 6·26; N, 28·7%. $C_4H_9O_3N_3$ requires C, 32·7; H, 6·12; N, 28·8%.)

N-*(γ-Glutamyl)hydroxylamine.* To hydroxylamine hydrochloride (5 g.) suspended in ethanol (20 ml.), 10 N-NaOH was added slowly and with shaking until the pH became 8 to 8·5. The mixture was filtered and the filtrate (20 ml.) with γ-ethyl glutamate (1·2 g.) was kept at room temperature in a closed vessel for 16 hr. and then at 0° for 48 hr., with shaking at intervals. The clear solution was then evaporated in a vacuum desiccator over P_2O_5 for 4 days, solid matter being removed by filtration each day. On the fifth day the volume of liquid was *c.* 3 ml. The pH was adjusted with concentrated HCl to 3·2 and the solution evaporated to a gum. The gum was rubbed with ethanol (40 ml.) and left at 0° overnight. The ethanol was decanted and the semi-solid mass taken up in water (10 ml.) and slowly precipitated as a pale-brown solid by the gradual addition of ethanol (50 ml.). Three recrystallizations from aqueous ethanol yielded pale buff-coloured needles (0·64 g., yield 57%; m.p. 155°), which gave a cherry-red colour with $FeCl_3$. (Found: C, 36·6; H, 6·11; N, 17·2%. $C_5H_{10}O_4N_2$ requires C, 37·0; H, 6·17; N, 17·3%.)

N-*(β-Aspartyl)hydroxylamine.* Asparagine monohydrate (5 g.) and hydroxylamine sulphate (5 g.) were boiled for 20 min. in water (25 ml.). The solution was cooled and a few drops of 10% (v/v) acetic acid added. Excess of saturated aqueous copper acetate was added gradually and the dark green precipitate filtered off and washed with water (200 ml.). The yield of copper salt was almost theoretical. The salt was suspended in 50% (v/v) aqueous ethanol and 1 drop of 0·1 N-H_2SO_4 added. H_2S was passed in and the mixture filtered at 60°. The pale-blue filtrate was repeatedly treated with H_2S until a clear colourless filtrate was obtained. The filtrate was evaporated to *c.* 5 ml. *in vacuo* at less than 50°. Ethanol (20 ml.) was added and the pale-brown precipitate was recrystallized three times from aqueous ethanol to yield off-white crystals (2·2 g., yield 45%; m.p. 179–180° with decomposition) which gave a cherry-red colour with $FeCl_3$. (Found: C, 32·1; H, 5·4; N, 19·2%. $C_4H_8O_4N_2$ requires C, 32·4; H, 5·2; N, 18·9%.) Estimation of the hydroxamic acid grouping by the method described below gave 97·5 ± 3% of the theoretical value.

Determination of hydroxamic acid grouping by $KBrO_3$. The method is based on that of Junell (1934) and consists of the oxidation of the hydroxamic acid grouping with $KBrO_3$, excess bromate being estimated by its oxidation of KI with subsequent estimation of liberated I_2. Solutions are required of $KBrO_3$ (0·04 M), $Na_2S_2O_3$ (0·025 M), HCl (N), KI (saturated aqueous), and of the hydroxamic acid (about 0·01 N). To the solution of hydroxamic acid (5 ml.) was added $KBrO_3$ (3 ml.) and N-HCl (2 ml.). Within 2 min. of the acidification excess KI solution was added and the liberated I_2 was estimated immediately with $Na_2S_2O_3$. The method gave consistent results to within 3% of the theoretical values. The time required for oxidation varied according to the hydroxamic acid being estimated. In the case of the two aminohydroxamic acids described above the oxidation was found (by spot testing with $FeCl_3$) to be complete within 1 min. under the conditions cited. For consistent results it was necessary to complete the estimation in less than 5 min. after acidification. Other hydroxamic acids (e.g. acethydroxamic acid) require up to 30 min. for complete oxidation.

α-Amino-γ-methylsulphinylbutyric acid. The method described is a modification of that used by Toennies & Kolb (1939).

DL-Methionine (10 g.) with glacial acetic acid (40 ml.) was warmed to 60° and then cooled to room temperature. Hydrogen peroxide (11 ml. of 100 vol.) was added slowly, the vessel being shaken and cooled between additions. The clear solution obtained was cooled to 0° and absolute ethanol (350 ml.) added. The mixture was cooled at 0° for several hours and the solid filtered off. The white amorphous powder (yield, 98%; m.p. 237° with decomposition) was washed with ethanol and dried *in vacuo*. Recrystallization from aqueous ethanol gave a white odourless crystalline solid (yield, 95%; m.p. 240° with decomposition). (Found:

C, 36·4; H, 6·63; N, 8·30; S, 19·4%. Calc. for $C_5H_{11}O_3NS$: C, 36·5; H, 6·68; N, 8·48; S, 19·4%.)

No attempt was made to separate the isomerides which are presumably present in this product, and which have been examined by Waelsch & Borek (1947).

α-Amino-γ-methylsulphonylbutyric acid. Preparation of this compound has been described by Toennies & Kolb (1941). The method below gives considerably poorer yield but was employed because of its simplicity.

DL-Methionine (4 g.) was suspended in 50% (v/v) acetic acid (20 ml.). $KMnO_4$ (45 ml. of 0·67 M in 50% (v/v) acetic acid) was added slowly and the solution was allowed to stand 15 min. at room temperature. H_2O_2 (100 vol.) was added dropwise until a clear solution was obtained. The straw-coloured solution was treated with charcoal, filtered, and ethanol (150 ml.) was added. The white precipitate was filtered off and recrystallized from aqueous ethanol. A white odourless crystalline solid (2·4 g., yield 53%; m.p. 254° with decomposition) was obtained. (Found: C, 33·3; H, 6·23; N, 7·6; S, 18·1, 17·5%. Calc. for $C_5H_{11}O_4NS$: C, 33·2; H, 6·08; N, 7·7; S, 17·7%.)

Compounds 1 *to* 8 *of Table* 1. These were all DL mixtures for which we are indebted to Dr J. Elks of Glaxo Laboratories Research Division.

Some microbiological properties of the compounds related to glutamic acid

The properties of the compounds related to glutamic acid have been investigated primarily in relation to haemolytic streptococci with results which are summarized in Table 1. Homologues of glutamic acid (compounds 1–6) and some other amino carboxylic acids (compounds 7 and 8) gave no instances of inhibition, of growth, of glutamine decomposition, or of glycolysis.

The two methionine derivatives proved inhibitory (see above) and the action of the *sulphoxide* (compound 9) on streptococcal growth is described by McIlwain *et al.* (1948). The *sulphone* (compound 10) had an entirely different action. Though without action on three streptococcal strains of varying glutamine requirement and also without action on a strain of *Staphylococcus aureus*, it inhibited growth of *Escherichia coli* in the medium based on ammonium lactate and described by Fildes (1938). The sulphone (0·02 M) prevented visible growth from an inoculum of *c.* 5×10^4 cells/5 ml. during the first 2 days and resulted in less growth than in controls during the following 2 days. These effects were prevented by the presence of 0·01 M-methionine and diminished by 0·002 M-methionine.

Hydrazine derivatives. The effect on streptococcal growth of the following compounds has been examined: γ-glutamylhydrazine and its acetone derivative; β-aspartylhydrazine, and hydrazine itself. Of these, the most inhibitory were hydrazine and γ-glutamylhydrazine. Hydrazine itself prevented growth of the Richards strain at 4×10^{-3} M and of the *faecalis* strain at 2×10^{-2} M, the concentrations in each case being independent of added glutamine. Inhibition by the glutamyl derivatives was, on the other hand, markedly influenced by the concentration of glutamine also present in growth media; the action of γ-glutamylhydrazine is described fully by McIlwain *et al.* (1948). Its acetone derivative acted similarly, but higher concentrations of it were required. Of the acetone derivative, 0·013 M (but not 0·01 M), prevented growth of the Richards strain for 3 days in the presence of 2×10^{-4} M-glutamine; with 2×10^{-5} M-glutamine, 5×10^{-3} M (not 3×10^{-3} M) was adequate. The benzaldehyde derivative was only slightly soluble, and at the concentrations which could be employed it was without action on growth. β-Aspartylhydrazine was required in concentrations of 4×10^{-2} M for inhibition of either the Richards or *faecalis* strains for more than 1 day, and in some cases this inhibition was diminished by either asparagine (0·01 M) or glutamine (2×10^{-4} M). Although the compound was not sufficiently active to investigate in detail, it indicates that the substitution of hydrazine by a residue similar to the glutamyl residue (as well as by the glutamyl residue itself) can produce a compound disturbing the action of glutamine. A less closely related compound, pantoylhydrazine (McIlwain & Hughes, 1945), has, it will be noted, a distinct type of action.

Hydroxylamine and hydroxamic acids. Growth of the Richards organism was partly inhibited by 4×10^{-5} M-*N*-(γ-glutamyl)hydroxylamine, and completely by concentrations of 2×10^{-4} M or greater, in the presence of either 2×10^{-6} or 2×10^{-4} M-glutamine. A *faecalis* and a group G strain were less sensitive to the compound, of which 2×10^{-3} M gave only a slight action, but this effect also was not altered by addition of glutamine. Hydroxylamine itself was inhibitory to streptococcal growth under the present conditions in concentrations of 5×10^{-5} M or more. To explain inhibition by the glutamyl derivative as due to hydroxylamine would require a considerable degree of breakdown, which model experiments showed should be detectable by reduction of Fehling's solution at room temperature. Such reduction was not found to be brought about by solutions of the glutamyl derivative; any hydroxylamine produced under the conditions of testing was found to represent only 0·01 mol. % of the glutamyl derivative used.

N-(β-Aspartyl)hydroxylamine was required in concentrations of 5×10^{-3} to 2×10^{-2} M, and *N*-acetylhydroxylamine in concentrations of 2×10^{-2} M or higher, for any inhibition of streptococcal growth. It was observed that the hydroxylamine derivatives reacted with iron salts of the growth medium to yield a yellow coloration (red in more concentrated solutions). Increase in iron content of the medium during growth did not however prevent the action of the inhibitors.

The determination of glutamine

Organisms. The Richards strain of β-haemolytic streptococci (National Collection of Type Cultures, no. 5631), passaged frequently through mice and of M.L.D. (in mice) of less than 100 cocci, was initially employed. Face masks were worn during the pipetting involved in photometric measurement of the growth of this organism. For the assay using methionine sulphoxide, a highly virulent organism was not required and this precaution was not taken. The same strain, after maintenance in laboratory media, and having a M.L.D. in mice of some 10^8 organisms, was then used. It was preserved by growing in serum broth for 7 hr., and drying *in vacuo* in a number of ampoules (cf. Hale & McIlwain, 1947). For use, a dried inoculum was grown in serum broth and from this, serum-broth-agar slopes were prepared daily for inoculation of the assay medium. Slopes were grown for 16 hr. and used only on the day following their inoculation; serum-broth cultures were kept in a refrigerator after growth and used only during the next 14 days. Incubation throughout was aerobic and at 37°. During preliminary studies, three other strains of β-haemolytic streptococci were examined as potential test organisms, but these offered no advantages over the Richards strain. As the specificity of this organism's response to glutamine in growth is already established (McIlwain, 1939), it has been used in the present study.

Medium and inoculation. A sterile solution of peptone, NaCl, KH_2PO_4 and NaOH was prepared according to McIlwain *et al.* (1939) and kept in a dark cupboard for periods of up to 1 month. For use, the following sterile solutions were added to each 100 ml. of peptone solution: the group A addenda of McIlwain (1944), 10 ml.; 0·2 M-methionine sulphoxide, 10 ml.; the yeast preparation of McIlwain (1944), 2 ml.; and N-NaOH, 2 ml. The medium was then inoculated with cocci taken from a serum-broth-agar slope; these were suspended in about 1 ml. of the peptone solution and the density of the suspension adjusted so that (by comparison with opacity standards) it contained approximately 10^8 organisms/ml. It was then diluted 1000-fold in the peptone solution and 2·5 ml. of the dilution added as inoculum. The inoculated medium was distributed in 5 ml. portions in a number of $6 \times \frac{3}{4}$ in. test tubes.

The inoculum chosen was sufficiently small not to require washing to free it from glutamine; a 5-fold increase or decrease in its size was tolerated without much effect on the assay.

Solutions containing glutamine, and incubation. 0·01 M-Glutamine was prepared at weekly intervals from the solid, sterilized by filtration through Ford's Sterimats in an apparatus of the type described by Knight & Fildes (1936) and kept cold. On the day of its use in assay, solutions were prepared from it which were 5×10^{-6}, 10^{-5}, $1·5 \times 10^{-5}$, 2×10^{-5} and $2·5 \times 10^{-5}$ M with respect to glutamine. Solutions to be assayed for glutamine were also filtered to sterilize them and diluted so that two or three dilutions could be expected to be within that range of concentrations. Known and unknown solutions, with the necessary control solutions (2 ml.) were then added to tubes of inoculated medium and incubated at 37° in 5% CO_2-air. An ordinary bacteriological incubator was used; shaking the tubes in a thermostat at 37° did not give a more regular response.

Measurement of streptococcal growth. This was carried out photoelectrically, using a Hilger absorptiometer to obtain the optical densities of the cultures after growth. While the conditions of assay were being chosen (i.e. in producing curves such as those of Fig. 1), portions of about 1·5 ml. of the cultures were taken at intervals, aseptically, with Pasteur pipettes. Cells were used which held *c.* 0·5 ml. of solution but gave an absorbing layer 1 cm. in depth. Densities were normally read after 16–24 hr. incubation, but sometimes also at 40 hr.; in the first case it was not necessary to take specimens aseptically.

Reference curves such as those of Fig. 1*B* were constructed from the readings obtained from the known quantities of glutamine. The glutamine content of each of the tubes containing solutions being assayed was computed by reference to the standard curve. Some results are quoted in Table 2. The standard curves often contained an almost linear portion as illustrated by the full lines in Fig. 1*B*, but sometimes were sigmoid, as shown by the broken line.

It will be observed that the assay could be made sensitive to 1/5 of the quantity of glutamine employed in the present account by using the same method of measurement but 1/5 of the quantity of media.

Specificity of assay. The effect of a number of compounds related to glutamine on growth of the present strain of streptococci has been examined (McIlwain, 1939), without finding any which replaced glutamine. The present medium already contains ammonium glutamate, and the addition of a further 2000 mμmol. of the substance per tube was found to have no effect on the assay of 20 mμmol. of glutamine. Glutamine added to bacterial extracts of the type assayed frequently by McIlwain *et al.* (1948) was satisfactorily recovered, indicating the absence of antagonistic or potentiating substances, but such experiments have not been performed with a wider range of materials.

Table 2 also gives results which show the value of the sulphoxide in assay. Values in the presence of the compound are seen to be much more consistent.

Table 2. *Results of glutamine assay, with and without sulphoxide and added glutamine*

Quantity taken of 1/50 dilution of horse plasma (ml.)	Glutamine found, calculated to nearest 10 mμmol./ml. plasma			
	By assay without sulphoxide		By assay with sulphoxide	
	Reading after 17 hr. growth	Reading after 25 hr. growth	Reading after 17 hr. growth	Reading after 25 hr. growth
2·0	830	710	530	490
1·6	840	830	520	500
1·2	730	770	520	520
0·8	690	750	510	510
Averages:	770	760	520	510

Quantity taken of		Glutamine found, calculated to nearest 0·1 mμmol./ml. extract			
		Reading after 18 hr. growth		Reading after 26 hr. growth	
Bacterial extract* (ml.)	Glutamine (mμmol.)	Directly	After subtracting quantity added	Directly	After subtracting quantity added
2·0	0	11·0	—	10·9	—
1·6	0	10·8	—	10·3	—
1·2	0	10·1	—	11·2	—
0·8	0	11·3	—	11·2	—
	Averages:	10·8	—	10·9	—
1·0	25	35·0	10·0	35·0	10·0
0·8	25	35·7	10·7	36·2	11·2
0·6	25	35·9	10·9	34·8	9·8
0·4	25	35·5	10·5	35·0	10·0
	Averages:	35·5	10·5	35·3	10·3

* Prepared by extracting *Staphyloccus aureus* (after treatment similar to that described by McIlwain *et al.* 1948; Table 10) with phosphate buffer pH 7 (4 ml./1·6 g. moist wt. of cells) at 100° for 15 min.; sulphoxide present during assay.

The reason for their absolute values being about 2/3 of those in the absence of the sulphoxide is not known. Acceleration of growth of the assay organism, by compounds unrelated to glutamine, might be expected to have a greater effect on the earlier than on the later readings, but the average values at the two times are found to be approximately equal. It is also possible that there exist in plasma substances which simulate glutamine in the absence of the sulphoxide, or promote its synthesis.

Measurement of response by acid formation. The assay organism converts glucose to lactic acid in a yield of *c.* 95 % (McIlwain, 1946). When this was measured by titration, a period of growth of some 3 days gave the most regular response to added glutamine (Fig. 2). As this period is longer than that required for measurement of growth by photometric readings, we have not attempted to base an assay on acid formation, although the type of response is adequate for such an assay.

Weight of streptococci produced by limiting quantities of glutamine. Assay curves such as those of Fig. 1*B* afford data which can be used in calculating the minimum quantity of glutamine required to produce a given quantity of streptococci. Comparison of the optical densities (*a*) and dry weights

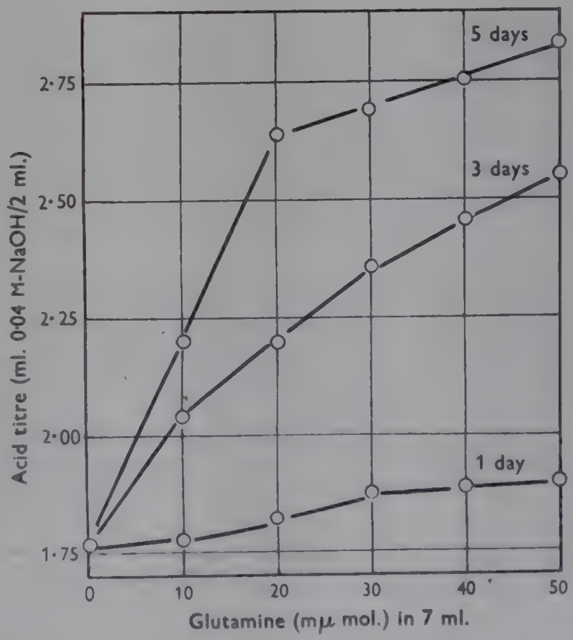

Fig. 2. Acid formed in streptococcal cultures initially containing varying quantities of glutamine, and measured by the alkali required at different times to take 2 ml. portions to a standard pH of about 11. Medium and conditions of growth: as described for photometric assay. Indicator: alizarin yellow G.

(b; mg./ml.) of different batches of the present organisms gave values for b/a of 0·368, 0·345, 0·377, 0·390 and 0·353 (mean: 0·367). The slopes of the initial portions of the lines of Fig. 1B show suspensions of optical densities 0·18 (in 16 hr.) to 0·23 (in 24 hr.) to be produced in the first day from 30 mμmol. of glutamine in 7 ml.; 0·015–0·02 mg. of organisms are thus formed per mμmol. of added glutamine. Eight other standard curves have given values differing from this by less than 25%.

SUMMARY

1. A number of substances structurally related to glutamic acid has been prepared, including the hydrazide, R.CO.NH.NH$_2$; its acetone derivative, R.CO.NH.N:C(CH$_3$)$_2$; its benzaldehyde derivative, R.CO.NH.N:CH.C$_6$H$_5$; the hydroxamic acid, R.CO.NHOH; and an analogous sulphoxide, R.SO.CH$_3$ and sulphone, R.SO$_2$.CH$_3$

(R = —CH$_2$.CH$_2$.CH(NH$_2$).COOH).

The hydrazide and hydroxamic acid similarly related to aspartic acid have also been prepared.

2. Some of these compounds, and also other substances related to glutamic acid (especially homologues of glutamic acid), had no effect on growth and glycolysis of streptococci. Those formulated above, except the benzaldehyde derivative and the sulphone (which however inhibited *Escherichia coli*), inhibited streptococcal growth. The basis for the action of the hydroxamic acid is unknown, but action of the other compounds on streptococci is related to the metabolism of glutamine or of glutamic acid.

3. Inhibition by the sulphoxide has been applied in the microbiological determination of glutamine. Glutamic acid can to some extent replace glutamine in growth of streptococci in ordinary media, but the sulphoxide (0·01 M) prevents this and results in a prompt and adequately stable growth of the organisms in the presence of 5–40 mμmol. of glutamine in 7 ml. of medium. Photometric measurement of the resulting growth has been used to determine glutamine in natural materials.

We are greatly indebted to Mr D. E. Hughes and Miss E. Ellis for assistance during these investigations.

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Relationships in Streptococci between Growth and Metabolism of Glutamine

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Previous investigation of the behaviour of glutamine as a growth essential for streptococci has suggested its action to have a metabolic basis in two senses. First, requirement for glutamine in growth appeared to depend on the inability of those streptococci which needed added glutamine to synthesize it at an adequate rate. This has now been confirmed by demonstrating the synthesis in organisms not requiring added glutamine, and further by inhibiting such synthesis when growth also has been found to be inhibited. Secondly, glutamine when added to streptococci has metabolic effects, among which has been recognized (McIlwain, 1946 *a*, *b*) a stimulation in glycolysis coupled with the conversion of glutamine to glutamic acid and ammonia. This group of reactions might be that for which glutamine (synthesized by the organisms or added as such) is required by streptococci. To previous evidence suggesting this has now been added two further types of observation: one, that a number of substances related to glutamine, but which are incapable of replacing glutamine in growth, have no effect on glycolysis; and the other, that when the coupled breakdown of glutamine is inhibited, growth of the organisms is also inhibited.

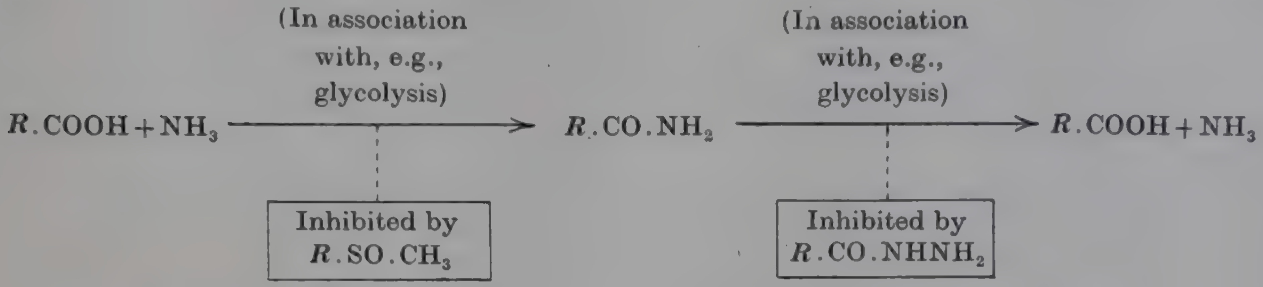
(In association with, e.g., glycolysis)
$R.COOH + NH_3$
$R.CO.NH_2$
(In association with, e.g., glycolysis)
$R.COOH + NH_3$
Inhibited by $R.SO.CH_3$
Inhibited by $R.CO.NHNH_2$

Fig. 1. Points of action of inhibitors in the synthesis and breakdown of glutamine. $R = -CH_2.CH_2.CH(NH_2).COOH$.

The reactions concerned are summarized in Fig. 1, and evidence is given below for their differential inhibition by two compounds structurally related to glutamic acid, namely, α-amino-γ-methylsulphinylbutyric acid, and γ-glutamylhydrazine. These compounds were selected for detailed study from a series described by Roper & McIlwain (1948). It will be observed from Fig. 1 that the synthesis and breakdown of glutamine are brought about by distinct systems, each associated with energy-yielding reactions such as glycolysis. Other aspects of the association between glutamine and glucose metabolism are reported below.

EXPERIMENTAL

Growth of streptococci, the performance of metabolic experiments with streptococcal suspensions, purification of glutamine, and determination of NH_3 and of glutamine were by methods previously stated (McIlwain, 1946 *a*, *b*) unless others are specified. Casein hydrolysate was that of McIlwain & Hughes (1944). The streptococcal strains employed are described by McIlwain (1946 *a*, *b*) and by Hale & McIlwain (1947).

Disintegration of bacteria followed Wiggert, Silverman, Utter & Werkman's (1940) method, using particles of Pyrex glass about the size of the bacteria. A mass of bacteria packed by centrifuging was cooled in ice and mixed with three times its (moist) weight of the glass, transferred to a dry, ice-cooled mortar and rubbed with maximum hand pressure for 30 sec., scraped together, and the rubbing twice repeated. The resultant paste was extracted in a centrifuge tube with cold M/18 phosphate, pH 7, 1·5 times the moist weight of the original bacteria. Disintegration was observed microscopically and its extent determined by mounting stained films in water instead of oil. The particles of glass could then be seen and the ratio of particles to bacteria was found before and after the grinding.

Extraction by autolysis. Organisms packed by centrifuging were suspended in seven times their moist weight of 0·055 M-phosphate of pH 7, containing 0·36 M-NaF, and left with occasional shaking at 37° for periods of 3–10 days.

Experiments with streptococcal extracts. In addition to the conditions of metabolism referred to above, a medium of the following composition (cf. Utter, Reiner & Wood, 1945) was used in attempting to obtain glycolysis in streptococcal extracts: Na_2HPO_4 and KH_2PO_4 of pH 7·3, 0·01 M; $NaHCO_3$, 0·05 M; $MgCl_2$, 0·008 M; glucose, 0·03 M; hexosediphosphate, 0·0025 M; adenosine triphosphate, 0·0006 M; cozymase (as a specimen of about 40% purity), 0·001 M, with and without nicotinamide, 0·04 M, and in equilibrium with 5% CO_2 in N_2.

Conditions employed in examining the actions of substances on streptococcal growth. A medium based on Bacto-peptone was used (McIlwain, Fildes, Gladstone & Knight, 1939; McIlwain, 1939), inoculated with about 500 cocci/ml. of the different streptococcal strains, and incubated at 37°. The mass of growth in control and experimental tubes was compared visually during the 7 days immediately following inoculation.

Materials. The compounds of Fig. 3 and Table 6 were either obtained commercially, or prepared according to McIlwain (1939). Hynson, Westcott and Dunning Inc., Baltimore, supplied bromsulphalein. Glutamine, glutamic acid and γ-glutamylhydrazine were the natural L-isomers. α-Amino-γ-methylsulphinylbutyric acid was the mixture of isomerides produced from DL-methionine by Roper & McIlwain (1948).

RESULTS

Effects of various circumstances on glutamine breakdown and on streptococcal glycolysis

Before comparing the behaviour of other compounds with that of glutamine, the effects of incidental circumstances on reactions between streptococci and glutamine itself were examined. The actions of glutamine which were studied were (*a*) the rate of glutamine breakdown, especially during anaerobic glycolysis, and (*b*) the associated stimulation of glycolysis.

Preparation of organisms. Results with organisms grown for different periods of time, and in different media, are given in Table 1. Though variations in the rate of NH_3 formation from glutamine occurred,

Table 1. *Variation in response to glutamine with age, medium of growth, and treatment of organisms*

(The Richards strain was employed, and cultures contained 5×10^{-4} M-glutamine unless otherwise indicated.)

Culture medium	Age (hr.)	Dry wt. used/3 ml. (mg.)	Q_{NH_3} (µmol./mg. dry wt./hr.)	Q_{CO_2} without glutamine (µmol./mg. dry wt./hr.)	Q_{CO_2} with glutamine as % of that without	Ratio $\frac{Q_{CO_2}}{Q_{NH_3}}$ in presence of glutamine
1. Casein hydrolysate	12	2·9*	2·55	25·5	126	12·6
2. Casein hydrolysate	26	2·8†	1·07	12·7	128	15·2
3. Broth (no added glutamine)	17	2·7	0·86	11·0	129	16·5
4. Glucose-broth	17	2·7	0·87	11·1	128	16·3
5. Casein hydrolysate; glutamine, 4×10^{-3} M	18	3·2	3·1	20·7	128	8·6
6. Casein hydrolysate; glutamine, 2×10^{-6} M	18	3·8	2·7	18·3	127	8·6
7. Casein hydrolysate; organisms washed once	17	5·0	1·95	15·3	124	9·7
8. Casein hydrolysate; organisms washed thrice	17	5·0	1·90	13·6	119	8·5
9. Casein hydrolysate; organisms washed thrice and incubated in saline‡	17	5·0	1·41	11·8	121	10·1
10. Casein hydrolysate; pyridoxin, 2×10^{-4} M	18	4·3	2·1	15·3	118	8·6

* Containing $2·7 \times 10^6$ viable organisms/mg. dry wt. † Containing $2·3 \times 10^5$ viable organisms/mg. dry wt.

‡ 10 mg. dry wt. of organisms in 10 ml. 0·9% (w/v) NaCl at 37° for 30 min.

these usually roughly paralleled the rates of glycolysis of the different preparations. This emphasized the dependence of glutamine breakdown on glycolysis; it was concluded that the different treatments did not have immediate effects on the reaction with glutamine. The conditions of growth included instances in which glutamine (medium 6, Table 1) or glucose (medium 3) were limiting factors in growth; organisms harvested at 12 hr. (medium 1) were at pH 7, while those harvested at 26 hr. (medium 2) had been at pH *c.* 5 for some hours. The effect of increased pyridoxin on growth was examined (medium 8) as a derivative of it acts as coenzyme in two reactions possibly relevant to glutamine (Gale & Epps, 1944; Gunsalus, Bellamy & Umbreit, 1944; Baddiley & Gale, 1945; Lichstein, Gunsalus & Umbreit, 1945) and the substance sometimes slightly accelerated growth of the 'Richards' streptococci in one of the experimental media, namely, that based on casein hydrolysate.

Concentration of glutamine in reaction-mixture, and time of reaction. The rate of NH_3 formation from glutamine during glycolysis by the 'Richards' streptococci (Table 2A) fell slightly during an hour's reaction, while the rate of glycolysis remained constant. In a separate experiment the streptococci were added to mixtures already containing glutamine and glucose; a relationship similar to that of Table 2A was observed, but with the superposition of short lags before glycolysis and glutamine breakdown reached their steady rates. The ratios of mol. NH_3 formed/mol. glutamine reacting did not markedly change during the experiment, but were between 0·88 and 0·95.

The fall in Q_{NH_3} observed at the end of the experiment of Table 2A was not due to the lowered concentration of glutamine which had then been reached. This is shown in Table 2B, where Q_{NH_3} is seen to be substantially unaffected by a 40-fold variation in glutamine concentration. The range of concentrations of the table cover those of blood plasma and tissues (cf. Archibald, 1945), but do not fall to the values at which glutamine becomes a limiting factor in streptococcal growth.

Table 2. *Kinetics of ammonia production from glutamine*

A. (Glutamine (8·3 µmol.) was added to glycolyzing streptococci (2·5 mg. dry wt.; Richards strain; final volume, 3·5 ml.) at 37° at a time taken as zero, in a series of vessels which were successively placed in ice at the times indicated. Values for Q_{NH_3} are calculated for the successive intervals.)

Time after mixing (min.)	NH_3 (µmol.)	Q_{NH_3} (µmol./mg. dry wt./hr.)
0	0	
15	1·67	2·6
25	2·66	2·4
38·5	3·84	2·1
53·5	5·32	2·3
70	6·50	1·7

B. (Organisms (Richards streptococci, 2·4 mg. dry wt.) were added at 37° to portions of the normal metabolic mixture containing the quantities of glutamine given below, in a final volume of 3 ml. After 34 min. the mixtures were cooled in ice and NH_3 determined. The values for mean concentrations of glutamine were calculated on the assumption that 1 mol. of glutamine disappeared for each mol. of NH_3 formed. See text, and McIlwain, 1946*b*.)

Initial glutamine (µmol.)	NH_3 formed (µmol.)	Mean glutamine concentration ($M \times 10^4$)
41·2	2·1	134
20·6	2·1	65
10·3	2·0	31·3
5·15	2·1	13·7
2·58	2·1	3·6

(Mean Q_{NH_3}, 1·53 µmol./mg. dry wt./hr.)

The effects of varying concentrations of glutamine on the duration and degree of stimulation caused in glycolysis have already been reported (McIlwain, 1946*a*).

Concentration of organisms in reaction mixture. This variable had a major effect on the stimulation of glycolysis. When, in the absence of added glutamine, a suspension of organisms was diluted so that the glycolyzing mixture contained *c.* 0·03 mg. dry weight of bacteria/ml. in place of *c.* 3 mg., their rate of glycolysis expressed as a metabolic quotient (i.e. after allowing for the decreased quantity of organisms) was lowered to about one-half. This occurred with organisms washed once and with others washed three times, and so was not dependent on the dilution of substances only loosely associated with the bacteria. The major effect of glutamine on glycolysis was to make good a large part of the deficiency induced by dilution (Table 3). The stimulation given by glutamine thus decreased with increasing concentrations of streptococci, as is illustrated in Fig. 2. The small stimulations in glycolysis quoted in Table 1 are associated with the relatively large concentration of organisms used in those experiments. As was shown previously (McIlwain, 1946*a*) the stimulation in glycolysis was not dependent on growth; optical density measurements during experiments of Table 3 were not found to change and so confirmed this.

In contrast with the results with streptococci comparable dilution of a suspension of an organism not requiring glutamine in growth did not affect its rate of glycolysis (Fig. 2).

The rates of decomposition of glutamine during glycolysis by 'Richards' streptococci in suspensions of density 1·5 and 0·075 mg. dry wt./ml. were compared, and found to differ by $<10\%$, though the stimulations of glycolysis in the two cases were markedly different (to 120 and 157% respectively of controls without added glutamine). Similar results were obtained with 'Buxton' streptococci in suspensions of densities 2·7 and 0·27 mg. dry wt./ml.

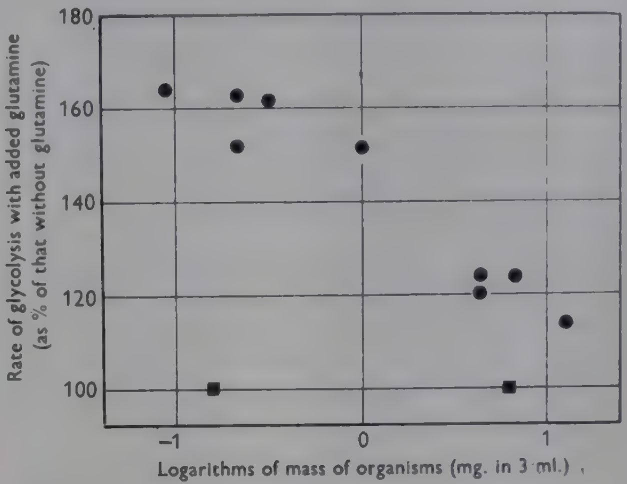

Fig. 2. Relationship between the effect of glutamine on glycolysis, and the concentration of cocci in the glycolyzing mixture. Circles, the Richards streptococcus. Squares, a strain of *Staphylococcus aureus* which did not require glutamine in growth.

Rates of glutamine breakdown and of glycolysis. Streptococcal glycolysis continues for some hours in the absence of added glutamine, though the organisms themselves contain relatively little glutamine (see below). There is therefore no necessarily fixed ratio between rates of glycolysis and of glutamine decomposition. When both substances are

Table 3. *Deficiency induced in glycolysis by dilution of bacterial suspension*

(Richards (R) or Buxton (B) streptococci were added at a time taken as zero to vessels containing the normal metabolic mixture with and without glutamine (20 μmol.) and of final volume 3 ml.)

		Q_{CO_2}				
		Without addition		With glutamine		
Organisms; dry wt. (mg. 3 ml.)	Change measured during period (min. after commencing reaction)	(μmol./mg. dry wt./hr.)	(As % of (*a*) in same experiment)	(μmol./mg. dry wt./hr.)	(As % of (*b*) in same experiment)	Ratio: $\frac{Q_{CO_2} \text{ with glutamine}}{Q_{CO_2} \text{ without glutamine}} \times 100$
R (batch 1) 13	0–30	10·8 (*a*)	100	11·7 (*b*)	100	109
R (batch 1) 1·08	0–30	7·1	66	10·6	90	150
R (batch 1) 0·09	0–120	4·5	42	7·5	64	167
R (batch 2) 4·4	5–25	18·1 (*a*)	100	22·3 (*b*)	100	123
R (batch 2) 0·22	5–25	14·6	80	21·6	97	148
R (batch 2) 4·4	25–45	17·8 (*a*)	100	21·4 (*b*)	100	120
R (batch 2) 0·22	25–45	11·6	65	19·2	90	166
R (batch 3) 7·7	0–25	18·1 (*a*)	100	21·7 (*b*)	100	120
R (batch 3) 0·38	0–25	11·1	61	18·2	84	164
B (batch 1 washed once) 1·2	0–115	9·35	—	13·0	—	139
B (batch 1 washed thrice) 1·2	0–115	9·20 (*a*)	100	9·60 (*b*)	100	104
B (batch 1 washed thrice) 0·06	0–115	4·7	51	7·0	73	149

present in excess, a relatively stable ratio between the two rates is however observed. The ratio is not markedly dependent on the conditions of growth and treatment of the organisms. Tables 1 and 4 show that values for a given strain vary, but not greatly when it is considered that the values quoted include experiments with independently grown organisms over periods of several months. Beyond such variation, differences characteristic of the strain of organisms can be discerned.

Table 4. *Rates of glutamine breakdown and of glycolysis by haemolytic streptococci*

(Reactions were carried out as described in Table 1; streptococcal strains were β-haemolytic with the exception of the *faecalis* strain.)

Strain; temperature of growth	Temperature of reaction	Q_{CO_2} (μmol./mg. dry wt./hr.)	Q_{NH_3} (μmol./mg. dry wt./hr.)	$\frac{Q_{CO_2}}{Q_{NH_3}}$
R; 37°	30°	9·6	1·28	7·6
R; 37°	37°	17·2	2·26	7·6
R; 37°	42°	20·6	2·55	8·1
R; 25°	37°	16·0	1·92	8·3
B; 37°	37°	6·7–13·1	0·81–2·01	4–14
UC2; 37°	37°	6·8–24	0·23–1·55	13–38
Strep. faecalis; 37°	37°	11·6–9·2	0·45–0·63	18–20·5
TI; 37°	37°	20·6	1·28	16·1

Tables 1 and 4 show no simple stoicheiometric relationship between the reactions in glucose and glutamine. The effect of glutamine in stimulating glycolysis may be expressed in terms of the additional CO_2 evolved while NH_3 is being formed from glutamine. No stoicheiometric relationship however exists between the NH_3 and additional CO_2 which are produced together. This reflects the observations above that (*a*) the percentage stimulation of glycolysis, and hence the additional CO_2, increases with decreasing concentrations of streptococci, while (*b*) the rate of NH_3 formation from glutamine does not markedly vary with concentration of organisms.

Attempted extraction of NH_3*-producing enzymes from streptococci*

Though Wiggert *et al.* (1940) obtained by ground glass from *Escherichia coli* extracts which were capable of glycolyzing, such an extract was not obtained from the Richards and group G streptococci. Rubbing which disintegrated 90–99 % of the cells was carried out and extraction of the ground material was performed at pH 5, 7 and 8. Further, the initial, packed, moist organisms were prepared by centrifuging suspensions in the reaction mixture with glucose, glutamine and coenzymes in order to protect such systems as could be stabilized by these substrates.

Both the extracts and residues of ground organisms were examined for their ability to decompose glutamine, but reaction was not observed. It was absent not only in the usual media with and without glucose, but also in more concentrated solutions, buffered with bicarbonate or phosphate, in which the minimum dispersion of enzyme systems took place. In such an experiment, 1 g. moist weight of strepto-

Table 5. *Arginine dihydrolase activity of streptococcal extracts; absence of glutaminase*

(The Richards streptococci were used, ground with glass, and extracted with 0·03 M-phosphate (pH 7). Reactions were carried out as described by McIlwain (1946*a*) in 3 ml. of phosphate saline at 37°.)

Extract; wt. used (mg.)	Dry wt. of bacteria corresponding to portion of extract used (mg.)	Substrate (μmol.)	Q_{NH_3} (μmol./equiv. dry wt./hr.)
A; 142	37	Glutamine, 25	0·02
A; 99	26	Glutamine, 5	0·02 ($Q_{glut.}$, −0·01)
A; 99	26	Nil	0·02
A; 189	50	Arginine, 20	0·92
B (first extract); 91	6	Arginine, 20	0·85
B (first extract); 91	6	Nil	0·02
B (first extract); 122, heated at 100° for 3 min.	8·2	Arginine, 20	0·02
B (second extract); 282	18	Arginine, 20	0·35

cocci was ground and extracted with 1·5 ml. of a solution containing glucose, bicarbonate, adenosine triphosphate, hexosediphosphate, inorganic phosphate, cozymase and glutamine. An extract (1·1 ml.) was obtained of which 0·25 ml. was mixed with 0·1 ml. further solutions for a metabolic experiment. Glutamine breakdown and glycolysis by the extract were <5 % of that of an equivalent quantity of whole organisms.

The inactivation of streptococcal enzymes by such disintegration was not a general one. The arginine dihydrolase (Hills, 1940) of such extracts was of special interest, as this enzyme leads to formation of NH_3, but independently of glycolysis. Extracts were obtained from the streptococci which were slightly opalescent and contained no cells, and which rapidly decomposed arginine but were without effect on glutamine (Table 5). The first extract, made at pH 7, had, at pH 7, one-third of the activity of the organisms from which it was prepared. About a further one-sixth of the original activity was obtained on a second extraction of the same cells. The remainder was not necessarily lost, as the pH optimum and other properties relating to the activity of the extracted enzyme have not been determined.

Autolysis did not yield from the streptococci an enzyme decomposing glutamine.

Specificity of the glutamine structure in relation to streptococcal glycolysis

Comparison has been made of a number of substances with glutamine with respect to (1) production of NH_3 during glycolysis, and (2) stimulation of glycolysis. Results of the comparison are given in Table 6 except in the cases of a few substances whose effects have been described previously (McIlwain, 1946*a*). Sixteen compounds were examined, using the Richards streptococcus, and a few with a group G organism.

The rate of NH_3 production from glutamine in control experiments was about 2 µmol./mg. dry wt./hr., and with the conditions adopted, production at a rate 1/50 of this could be detected. No material yielded NH_3 at a rate of $>0·05$ µmol./mg. dry wt./hr. with the exception of arginine and mixtures containing it; this has been reported previously and, as is noted above, occurred independently of glycolysis (Hills, 1940; McIlwain, 1946*a*).

Stimulation of glycolysis to the extent caused by glutamine was not produced by any compound examined. It was reported (McIlwain, 1946*a*) that NH_3 itself caused a lesser stimulation, and that a few compounds, known or presumed to produce NH_3 under the conditions of the present experiments, also

Table 6. *Effects on streptococcal glycolysis, and on* NH_3 *production, of compounds related to glutamine and to* NH_3

(Organisms (0·5–1 mg. dry wt.) were added to reaction mixtures containing glucose and bicarbonate (final vol., 3 ml.); conditions anaerobic.)

Reaction mixture		Q_{CO_2} as % of the value without addenda, for the same period		
Organism	Addenda (µmol.)	0–30 min.	30–60 min.	Mean Q_{NH_3} (µmol./mg. dry wt./hr.)
	A. Ammonia and simple amines			
R	NH_4Cl, 25	110–118	110–116	—
R	NH_4Cl, 2·5	108–116	107–115	—
R	CH_3NH_3Cl, 25	102	103	—
R	CH_3NH_3Cl, 2·5	103	104	—
R	$(CH_3)_2NH_2Cl$. 25	92	95	—
R	$(CH_3)_2NH_2Cl$, 2·5	103	97	—
R	$C_2H_5NH_3Cl$, 25.	103	97	—
	B. Glutamine and related compounds			
R, G	Glutamine, 2·5–50	140–175	140–180	+1·5–2·5
R, G	L- or DL-Glutamic acid, 2·5–15	97–103	100–106	$<0·05$
R	Glutaric acid monoamide, 50	101	105	$<0·03$
R	α-Acetamidoglutaric acid amide, 10, 50	99, 102	96, 105	$<0·03$
R	DL-Uramidoglutaric acid, 2·5–15	97	98	$<0·04$
R	Pyrrolidone-α-carboxylic acid, 2·5, 15	96	100	$<0·03$
R	Pyrrolidone-α-carboxylic acid amide, 2·5, 15	104	102	$<0·04$
	C. Other amino-acids			
R, G	Asparagine, 25	101	105	$<0·03$
R	Aspartic acid, 25	96	98	$<0·04$
R	Proline, 2·5, 20	103	104	$<0·03$
R	Citrulline, 20	101	114	*c.* 0·05
R	Arginine, 10	104	117	+3·9

stimulated glycolysis to values approximating to those of NH_3. To such compounds may now be added citrulline, though the rate of NH_3 production from this compound was small (Table 6). Dimethylamine and ethylamine were without effect. The action of methylamine may be due to NH_3 in the specimen, which was not especially purified; it can be concluded that any effect of methylamine is less than that of NH_3.

The action of inhibitors on glutamine breakdown, glycolysis and on growth

The effect of further substances on these three processes has been examined to see to what extent the response of the processes to given circumstances are correlated. Correlation would suggest their functional interrelation.

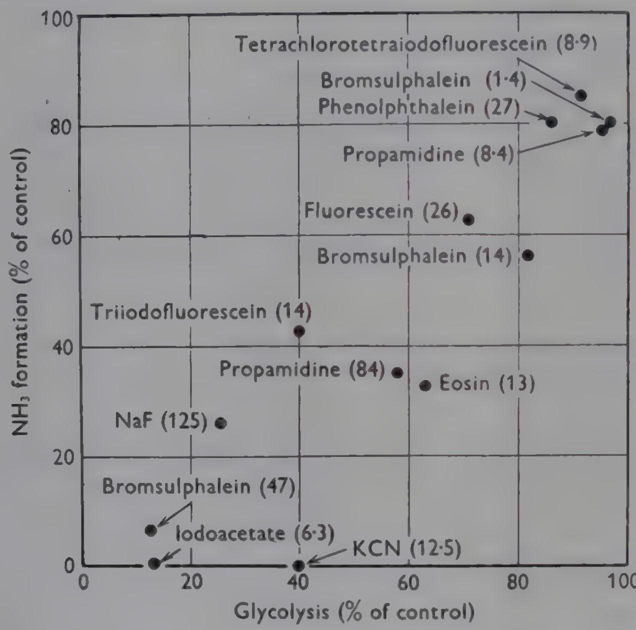

Fig. 3. Comparison of the effects of several inhibitors on streptococcal glycolysis in the presence of glutamine, and on the associated production of NH_3 from the glutamine. The Richards strain was used throughout; numbers beside the names of the inhibitors give their molarities ($\times 10^4$).

Phthaleins. Glutamine hydrolysis by streptococci has not yet been brought about in the absence of glycolysis; in previous attempts to do so by addition of substances inhibiting glycolysis, the glutamine breakdown was also prevented (McIlwain, 1946*a*). As bromsulphalein (a sodium salt of a disulphonic acid of tetrabromophenolphthalein) inhibits a glutaminase of animal tissues (Archibald, 1945), which is not described as being dependent on carbohydrate metabolism, its effect on glutamine metabolism in streptococci has been studied. Bromsulphalein has been found to inhibit the breakdown of glutamine by streptococci, but it inhibited also both glycolysis and growth, often when present in relatively low concentrations. Phenolphthalein, fluorescein, eosin, and a triiodofluorescein were also potent inhibitors of both glycolysis and the breakdown of glutamine in streptococci (Fig. 3).

When the concentrations of the various substances which affect glycolysis and glutamine metabolism were compared, as in Figs. 3 and 4, glutamine breakdown was seen to be the more sensitive of the two processes. It was however the more sensitive to all reagents examined, which showed any selectivity between the two reactions; the reagents studied included monoiodoacetate, cyanide and propamidine. The results of Figs. 3 and 4 thus emphasize the general connexion between glutamine metabolism and glycolysis, without giving any certain evidence that the phthaleins might affect glycolysis through an action on glutamine metabolism.

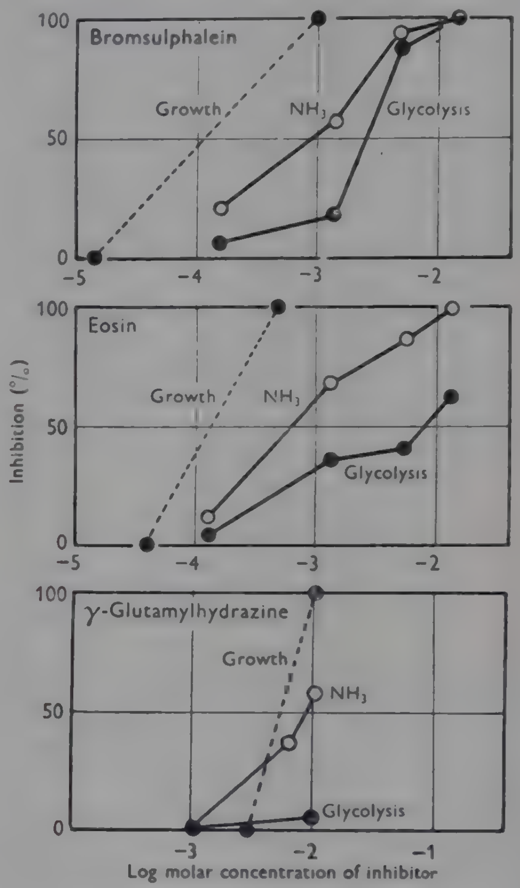

Fig. 4. Inhibition of growth, of the formation of NH_3 from glutamine, and of glycolysis, by different concentrations of bromsulphalein, eosin, and γ-glutamylhydrazine. Growth was studied as described in Table 7, and metabolism as described in Table 1.

The phthaleins inhibited also the growth of streptococci, and the effect of varying concentrations of eosin and bromsulphalein on growth was examined. Growth is seen (Fig. 4) to be somewhat more sensitive to these compounds than are glycolysis and glutamine decomposition. The values given in Fig. 4

for the concentrations of the substances affecting growth are approximate only, referring to those of a series of 3-fold dilutions which inhibited completely, or were without visible effect on, growth. They are however adequate to show a marked difference in character of action between the phthaleins and γ-glutamylhydrazine which is discussed below.

Table 7. *Action of varying concentrations of γ-glutamylhydrazine on streptococcal growth*

(Growth was described as prevented if visible opacity did not develop during the 3 days following inoculation under conditions described in the text. Little change was observed during the next 4 days.)

Strain	(*a*) Concentration of glutamine initially present in medium (M)	(*b*) Concentration (M) of glutamylhydrazine		Ratio *b*/*a*	
		Minimal to prevent growth	Maximal allowing growth	Preventing growth	Allowing growth
RNI	2×10^{-3}	10^{-2}	5×10^{-3}	5	2·5
RNI	2×10^{-4}	$2{\cdot}5\text{–}5 \times 10^{-3}$	$1{\cdot}25\text{–}2{\cdot}5 \times 10^{-3}$	12·5–25	6·3–12·5
RNI	2×10^{-5}	$1{\cdot}25\text{–}2{\cdot}5 \times 10^{-3}$	$6{\cdot}25\text{–}10 \times 10^{-4}$	62·5–125	31–50
L	2×10^{-3}	—	2×10^{-2}	—	10
L	2×10^{-4}	2×10^{-2}	10^{-2}	100	50
L	2×10^{-5}	5×10^{-3}	$2{\cdot}5 \times 10^{-3}$	250	125
Strep. faecalis	2×10^{-4}	4×10^{-2}	10^{-2}	200	50
Strep. faecalis	None added	10^{-2}	$2{\cdot}5 \times 10^{-3}$	—	—

The following experiments were carried out in attempting to see whether inhibition of growth by the phthaleins was related to glutamine. Inhibition of the Richards streptococci by bromsulphalein was compared in the presence of minimum glutamine for maximal growth, and in a concentration of 20 times that value. No marked difference was seen. Growth of a group G organism not requiring added glutamine was also inhibited by bromsulphalein, and the level required for inhibition was not affected by the presence of 10^{-3} M-glutamine. Tetrachlorotetraiodofluorescein behaved similarly to bromsulphalein in these two respects.

Effects on growth of compounds structurally related to glutamine. It was anticipated that such compounds might afford examples of inhibition connected with the metabolism of glutamine and of value in studying its effect on growth. Of the many substances examined (Roper & McIlwain, 1948) γ-glutamylhydrazine exhibited a type of action on growth which was promising from this point of view.

Results quoted in Table 7 showed several streptococcal strains to be inhibited by the hydrazide at concentrations of 6×10^{-4} to 2×10^{-2} M. The organisms of Table 7 required added glutamine to varying degrees (see Table 12) but the growth of all could be inhibited by the hydrazide; more of this was however required for inhibition of the less exacting *faecalis* or L strains. Inhibition by the hydrazide was in all cases antagonized by increasing the concentration of glutamine present in the growth medium. The Richards and L strains were examined sufficiently to show that, with increasing concentrations of glutamine in growth media, increasing concentrations of the hydrazide were needed to effect a given degree of inhibition: the hydrazide competed with glutamine. The concentration of hydrazide required for a given effect did not however increase in direct proportion to the increase in concentration of added glutamine. The ratios of Table 7 (and also Fig. 5 which is discussed in detail below) show that a greater preponderance of the hydrazide is needed to inhibit the lower concentrations of glutamine.

The compound had no immediate bactericidal action (Table 8). As its action on growth was associated with glutamine, its effects on the metabolism of glutamine were examined.

Table 8. *Absence of immediate bactericidal action by γ-glutamylhydrazine and α-amino-γ-methylsulphinylbutyric acid*

(The L strain of streptococci grown for 16 hr. was diluted in buffered casein hydrolysate.)

Substance added (M)	Colonies/$1{\cdot}5 \times 10^{-4}$ ml. at times		
	0 hr.	2 hr.	6 hr.
Nil	475	317	244
γ-Glutamylhydrazine (0·02)	—	313	285
α-Amino-γ-methylsulphinylbutyric acid (0·02)	—	348	255

Action of γ-glutamylhydrazine on glycolysis and on the production of NH_3 from glutamine. The type of experiment which was performed is illustrated by Table 9. From this, it can be seen that the hydrazide was effective as an agent inhibiting the formation of NH_3 from glutamine by a variety of streptococcal strains. Its action on the glycolysis with which the glutamine breakdown is associated was much less. In several instances, while NH_3 production was

Table 9. *Action of γ-glutamylhydrazine on glycolysis and breakdown of glutamine by streptococci*

(Warburg vessels contained the quantity of hydrazide needed to give the concentrations tested, with bacteria (c. 3 mg. dry wt.), salts and $NaHCO_3$ (0·06 M) in 2·0 ml. of water; glutamine (in 0·5 ml. water) was added from a side arm after equilibration with 5 % CO_2 in N_2. A centre well contained yellow phosphorus.)

	Reaction mixture			Products	
Strain	(*a*) Glutamine (M)	(*b*) Hydrazide (M)	Ratio *b*/*a*	Acid formation as % of control without added glutamine and inhibitor	NH_3 formation as % of control with glutamine but without inhibitor
R	4×10^{-3}	2×10^{-2}	5	95	42
R	4×10^{-3}	$6{\cdot}6 \times 10^{-3}$	1·67	102	62
R	4×10^{-3}	2×10^{-3}	0·5	97	96
R	4×10^{-3}	0	0	131	100
L	4×10^{-3}	2×10^{-2}	5	99	14
L	4×10^{-3}	$6{\cdot}6 \times 10^{-3}$	1·67	110	50
L	4×10^{-3}	2×10^{-3}	0·5	113	100
L	4×10^{-3}	0	0	119	100
L	$1{\cdot}6 \times 10^{-2}$	$6{\cdot}6 \times 10^{-3}$	0·42	116	100
Strep. faecalis	$3{\cdot}3 \times 10^{-4}$	$2{\cdot}2 \times 10^{-2}$	6·7	95	33
Strep. faecalis	$3{\cdot}3 \times 10^{-4}$	Methionine sulphoxide $3{\cdot}3 \times 10^{-2}$	—	108	105
Strep. faecalis	$3{\cdot}3 \times 10^{-4}$	0	—	108	100

lowered considerably, glycolysis was lowered only to a value about that found in the absence of glutamine.

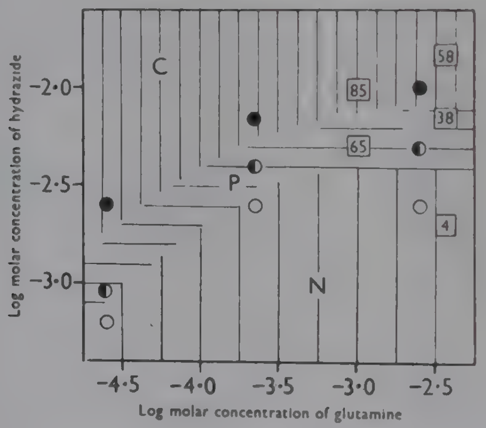

Fig. 5. Comparison of the degrees of inhibition caused in growth and in the formation of NH_3 from glutamine (streptococcus R) by γ-glutamylhydrazine. Circles and shaded areas represent the effects on *growth* of varying concentrations of the hydrazide in the presence of varying concentrations of glutamine. The figures in squares give the percentage inhibition caused in the *formation* of NH_3, at the concentration of glutamine represented by the abscissa value of the square, by the concentration of hydrazide represented by its ordinate value. C and ●, complete; P and ◐, partial; N and ○, no inhibition of growth.

The process of NH_3 formation from glutamine is sensitive to about the same range of hydrazide concentrations, as is growth. Comparison of some of the values of Tables 7 and 9 shows this, and the two processes are compared directly in Fig. 4, which shows that an inhibition of 50 % in the rate of NH_3 formation is associated with a marked inhibition of growth. Fig. 4, in comparing the action of γ-glutamylhydrazine on glycolysis, NH_3 formation and growth, indicates that it has a markedly different type of action from that possessed by the phthaleins and supports the suggestion that the action of the latter compounds on glutamine breakdown is a relatively indirect one.

The substance had little if any action on the assimilation of glutamic acid and glutamine (see below, and Table 11).

Comparison of the action of the hydrazide on growth and on glutamine decomposition. As the major metabolic effect which we have recognized as being brought about by γ-glutamylhydrazine is its action on the formation of NH_3 from glutamine, we have carried out a more extensive comparison between its inhibition of that process and its inhibition of growth. Both inhibitions are affected by the concentration of glutamine present, and are therefore represented in the fashion of Fig. 5. Here it is seen that in mixtures of the hydrazide and glutamine in which the decomposition is inhibited, growth is affected; 40–60 % inhibition of the decomposition is associated with partial inhibition of growth, and 60–85 % with complete inhibition of growth.

Glutamine synthesis by streptococci. Inhibition of the synthesis, and of growth of the organisms

Demonstration of the synthesis. It has been presumed that streptococci not needing glutamine in growth synthesize the compound, though the synthesis has not previously been demonstrated

(McIlwain *et al.* 1939; Fildes & Gladstone, 1939). Attempted determination of glutamine by NH_3 produced on hydrolysis gave negative results with culture fluids, but showed a small quantity of a glutamine-like substance to be associated with streptococcal cells. In order to study changes in the quantity of such material, the microbiological method of determining glutamine which has been described by Roper & McIlwain (1948) was developed. It was then found that a substance, possessing some chemical and microbiological properties of glutamine, could readily be obtained from streptococci grown in the absence of added glutamine. They had therefore synthesized it.

For further study it was desired to bring about the synthesis under conditions more defined than those of a growing culture. Washed streptococcal cells exposed to ammonium glutamate did not yield a glutamine-like substance; but of other materials present during the synthesis by growing cells, glucose, when added with ammonium glutamate, was found to result in the formation of a glutamine-like substance. Experimental conditions for the synthesis are given in Table 10, together with a comparison of the properties of the product with those of glutamine. These properties, the conditions of production of the substance, and its consistent determination as glutamine by a method which itself is highly specific to glutamine make it probable that the material present in the cells is glutamine itself. Of other compounds, those known to be of the lability indicated in Table 10, and perhaps most likely to be present in the cells, are peptides of glutamine such as were studied by Melville (1935); these have however been found not to replace glutamine in growth of the assay organism (McIlwain, 1939). It can be seen from Table 10 that some 10 % of glutamine is probably lost during the treatment employed for extracting it from streptococci. No correction for such loss has been applied to the results quoted in this paper, but the conditions of extraction have been rigidly adhered to and applied to washed cells; results have been found to be reproducible to within the limit of accuracy of the assay (about 4 % of their value).

Table 10. *Properties of a glutamine-like substance produced by streptococcal cells*

(The material was obtained from 6·5 g. moist wt. of *Streptococcus faecalis*. The cells were shaken aerobically (anaerobiosis made little change in the result) with ammonium L-glutamate (0·16 mmol.), glucose (0·5 mmol.) and Na and K phosphates (1 mmol.) initially at pH 7·6, in 50 ml. water at 37° for 22 min., at which time the pH had become 6·8–7; the cells were washed and the material extracted from them by heating at 100° for 10 min. in 10 ml. 0·005 M-Na and K phosphates at pH 7. It was centrifuged free from cells and contained the equivalent of 160 mμmol. glutamine/ml. Untreated cells extracted in the same way yielded a solution containing 18 mμmol./ml. of glutamine. The suspension of *Clostridium welchii* was prepared and used according to Gale's (1945) description.)

Treatment	Material undergoing treatment	Initial concentration (expressed as glutamine (M))	Material remaining (% of initial quantity)
10 min. at 100°, pH 7	Glutamine	10^{-4}	85–90
	Extract	10^{-4}	90–95
1 hr. at 100°, pH 6, in 0·2 M-phosphate	Glutamine	$1{\cdot}6 \times 10^{-4}$	42
	Extract	8×10^{-5}	32
	Glutamine	8×10^{-5}	30
	With extract	8×10^{-5}	
20 min. at 100° in N-H_2SO_4	Glutamine	8×10^{-5}	<5
	Extract	8×10^{-5}	<5
30 min. at 37° and pH 4·5 with suspension of *Cl. welchii*	Glutamine	10^{-4}	<4
	Extract	8×10^{-5}	<4

Kinetics. The assimilation of glutamic acid and glutamine. Gale (1947) has shown that an energy-yielding reaction such as glycolysis is required for entry of glutamic acid or glutamine into streptococcal cells, and during the present experiments ample confirmation of these observations has been found. The rate and extent of such assimilation is clearly relevant to glutamine synthesis and we have, therefore, followed assimilation while studying glutamine synthesis. Total glutamic acid and glutamine have been determined by the decarboxylase preparation from *Clostridium welchii* (Gale, 1945). Though this preparation liberates CO_2 from glutamic acid and from glutamine at different rates, we did not find the difference adequate to form the basis of a method for determining glutamine in the presence of glutamic acid in streptococcal extracts. The reagent was therefore used in quantity sufficient to determine both substances, and glutamine determined separately by the method described above. The quantity of glutamine found in cells was usually a fraction only of the quantity of glutamic acid. Both determinations were made after the extraction described in Table 10.

Several experiments on the course of entry of glutamic acid and glutamine are illustrated in Fig. 6. In these, glutamic acid was usually supplied together with 1 equiv. of NH_3, at concentrations between 8×10^{-3} and 5×10^{-4} M. Initial concentrations of glutamine were similar. Glucose was usually present in excess of the quantity metabolized in the 20–45 min. experimental period, and phosphate buffer in quantity adequate to maintain the pH between 7·6 and 7·0. The quantity of streptococci used was between 3 and 10 mg. dry wt./ml. of reaction mixture, but in Fig. 6 results are expressed as the quantity of glutamic acid or glutamine entering unit weight of cells at various times; the slopes of the lines joining experimental points therefore correspond to metabolic quotients.

Entry of glutamic acid and glutamine into the *faecalis* strains are compared in Fig. 6. The initial rates of entry which were observed in the two compounds were similar and corresponded to between 0·2 and 0·5 μmol./mg. dry wt./hr. The experimental arrangements were such that these were not necessarily the maximal rates of which the organisms were capable, as the first reading was commonly taken after 8 min. exposure, and between it and the second reading a marked fall in rate of assimilation frequently occurred. A smooth curve through the experimental points of Fig. 6 suggested maximal initial rates approaching 1 μmol./mg. dry wt./hr. in the presence of initial concentrations of glutamic acid or glutamine of $5{\cdot}5\text{–}8 \times 10^{-3}$ M. During assimilation, glycolysis was proceeding at the rate of about 10–15 μmol./mg. dry wt./hr. (Table 11). Rate of entry varied with the concentration of added glutamic acid, in the manner indicated in Table 11. The initial rate of assimilation of glutamic acid from a solution $5{\cdot}5 \times 10^{-3}$ M was not found to be affected by increase of the concentration of ammonium ion from 5·5 to $27{\cdot}5 \times 10^{-3}$ M. The final concentration of glutamine plus glutamic acid which was reached in cells was frequently greater when glutamine was supplied than when ammonium glutamate was used. This is seen in two instances in Fig. 6, and was most often found at the end of experiments in which the supply of glucose was failing.

Kinetics. The breakdown of glutamine during assimilation experiments. Streptococci rapidly break down glutamine when this is added to them in the presence of glucose. Before studying the possible

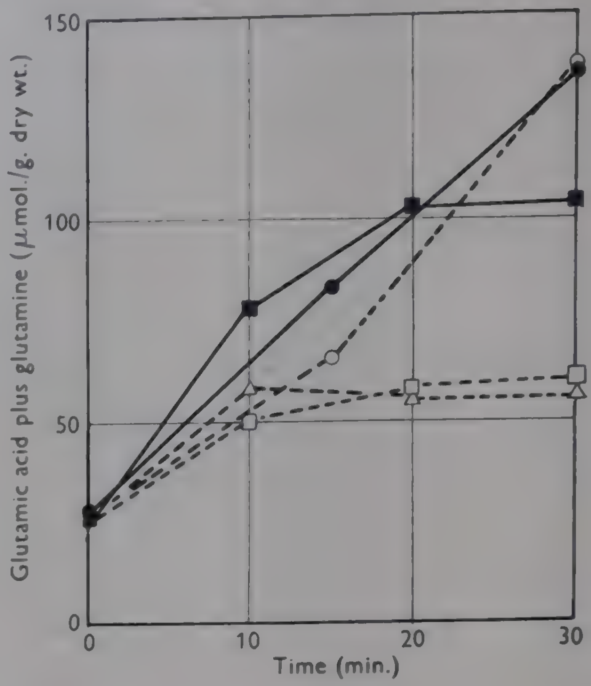

Fig. 6. The course of entry of glutamic acid and glutamine into three batches of cells of *Streptococcus faecalis.* Suspensions of bacteria were divided into portions containing *c.* 0·5 g. wet weight of cells, and these shaken at 37° with 15–20 ml. of 0·08 M-Na and K phosphates of initial pH 7·5 and containing 0·017 M-glucose and 0·008 M-ammonium glutamate or glutamine. Cells were separated after the times indicated, washed, and their content of glutamic acid plus glutamine compared with that of a sample of untreated cells, after the extraction of Table 10. Full lines and black points, uptake following addition of glutamine. Broken lines and white points, uptake following addition of glutamic acid. Points of the same shape refer to experiments carried out simultaneously and with the same batch of cells.

Table 11. *Assimilation of glutamic acid from different initial concentrations; the course of assimilation and of lactic acid formation*

(The organisms and reaction mixtures (18 ml.) were as described in Fig. 6. Organisms *A* contained initially 1·7 μmol., organisms *B*, 2·1 μmol. and organisms *C*, 1·35 μmol. of glutamic acid in the quantity of cells employed below; each contained <0·02 μmol. of glutamine.)

Batch and dry wt. of streptococci (mg.)	Glutamic acid added		Glutamic acid assimilated (μmol.) in *t* min.		Glutamic acid remaining in solution (by difference)		Lactic acid formed
	(μmol.)	(M × 10^{-4})	(*t*)	(μmol.)	(μmol.)	(M × 10^{-4})	(μmol.)
A, 120	54	30	8	3·75	50·2	28·0	294
A, 120	18	10	8	2·40	15·6	8·7	315
A, 120	9	5	8	2·00	7·0	3·9	—
A, 120	0	0	8	—	—	—	289
B, 168	100	62·5	8	1·35	99	55·0	223
B, 168	100	62·5	15	1·95	98	54·5	468
B, 168	100	62·5	30	2·30	98	54·5	502
C, 188	50	—	10	1·35	48·6	27·0	342
C, 188	50; with glutamylhydrazine, 500	30	10	1·45	48·5	26·9	322
C, 188	0	—	10	0	0	0	349
C, 188	0; with glutamine, 50	0	10	2·95	—	—	410
C, 188	0; with glutamine, 50, and glutamylhydrazine, 500	0	10	2·75	—	—	352

synthesis of glutamine, experiments were carried out to see whether any accumulation of glutamine within the cells could be demonstrated when the compound itself was added. Transitory accumulation has been found. Fig. 7 shows this. Here the concentration of glutamic acid plus glutamine rises during the first 20–40 min., and does not fall during the experimental period. Glutamine itself, however, falls in concentration after 10 min. Short experiments are therefore likely to be necessary to demonstrate its synthesis.

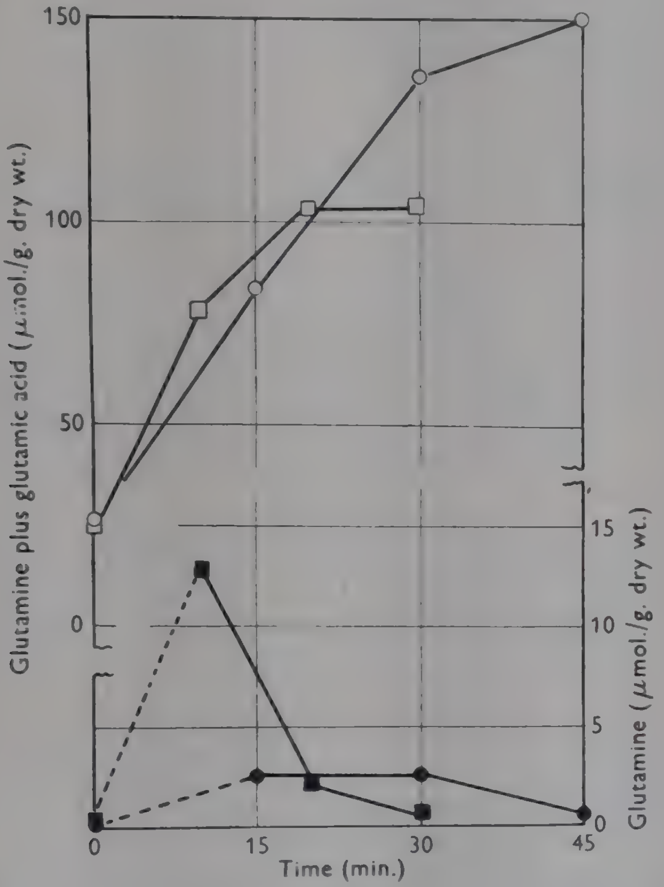

Fig. 7. The temporary accumulation of glutamine, and appearance of glutamic acid, in *faecalis* streptococci exposed to glutamine and glucose. Experimental arrangement, as described in legend to Fig. 6. Black points, glutamine. White points, glutamic acid plus glutamine. Points of the same shape refer to experiments carried out simultaneously and with the same batch of cells.

Kinetics. The synthesis of glutamine from ammonium glutamate. Fig. 8 shows incubation of *faecalis* streptococci with glucose and ammonium glutamate to result in a rise and then a fall of intracellular glutamine. The maximum quantity observed was only 1 mol. % of the ammonium glutamate added and 3 mol. % of the glutamic acid present in the cell at the same time. These values are to be compared with those of Fig. 6 describing the behaviour of added glutamine. Values for the same batch of organisms are available, and here the maximum accumulation observed was 8·5 % of the glutamine added, and 16 mol. % of the intracellular glutamic acid. Synthesis is thus slower than is the entry of glutamine, but it is not known what reduction in the rate of entry would be necessary to give the observed accumulation. The maximum rate of accumulation which we have observed in synthesized glutamine is 0·02 μmol./mg./hr., in cells capable of decomposing added glutamine at the rate of 0·6 μmol./mg./hr.

Connexion between the accumulation of synthesized glutamine in several streptococcal strains and their need for added glutamine in growth. Synthesis of glutamine has been observed in many streptococcal strains in addition to those of Fig. 8. In most cases accumulation was not followed kinetically, but the maximum values which have been observed during short experimental periods are quoted in Table 12. They are seen to vary inversely with the organisms' need for glutamine in growth. This need has been expressed as the increased lag observed in their growth, following the withdrawal of glutamine and the substitution of ammonium glutamate; details are given elsewhere (Hale & McIlwain, 1947). When so expressed organisms most exacting with respect to glutamine gave a value for increased lag (a totalled value under a set of arbitrarily chosen conditions) of about 35 days, and organisms not requiring added glutamine gave a value of zero.

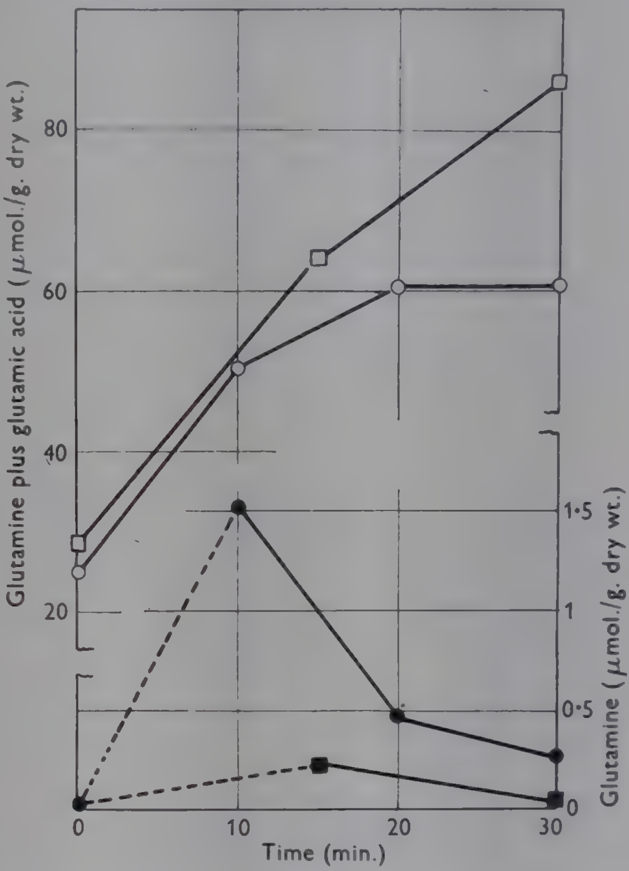

Fig. 8. Accumulation of synthesized glutamine by *faecalis* streptococci. Experimental conditions, as legend to Fig. 6, employing ammonium glutamate with no added glutamine. Symbols, as legend to Fig. 7.

Table 12 shows that the exacting organisms accumulated glutamine at a very slow rate, if at all; while non-exacting strains showed marked synthesis.

Table 12. *Variation in the accumulation of synthesized glutamine by several streptococcal strains*

(Organisms were grown in 1 or 0·5 l. of the medium based on casein hydrolysate and yeast preparation, and, in order to ensure uniformity of growth conditions, glutamine (10^{-5} M) was present in all cases although several organisms did not require its addition for their growth. At 14–24 (usually 16) hr., cells were harvested, washed twice in saline and portions representing about 0·5 g. wet wt. of organisms were used in 16 ml. of solution which contained ammonium glutamate, 0·003 M; glucose, 0·0125 M and phosphates at pH 7·6, 0·085 M. The mixtures were shaken aerobically at 37° and at 10 and 20 min. were cooled with ice and the cells separated and washed with saline. Their cell contents were freed as described in Table 10 and glutamine determined in the extracts. From values so obtained were subtracted the small quantities of glutamine initially present in the cells and determined on another portion which had been kept cold during the experiment. In instances in which it was examined, the solution after exposure of the cells to ammonium glutamate, contained no glutamine.)

Streptococcal strain	Lag on withdrawing glutamine (arbitrary total, days)	Maximum observed rate of accumulation of glutamine (μmol./g./hr.)
6176; β-haemolytic	31	<0·1
RNI; β-haemolytic	26	<0·2
6177; β-haemolytic	8	<0·1
L; β-haemolytic	3	0·3
6181; β-haemolytic	0	2·6
Faecalis; laboratory strain	0	5·3
Faecalis; N.C.T.C. 6796	0	2·2

Inhibition of growth by methionine sulphoxide. Of the compounds examined by Roper & McIlwain (1948), one was initially found to inhibit the growth of a *faecalis* strain of streptococci but not that of the Richards strain. This was α-amino-γ-methylsulphinylbutyric acid, the sulphoxide derived from methionine. The basis for its differential action was found to lie not in the organisms examined but in their conditions of growth; the *faecalis* strain, being non-exacting towards glutamine, was grown without added glutamine while the Richards strain was necessarily grown with glutamine. When the *faecalis* strain was grown in the presence of glutamine, α-amino-γ-methylsulphinylbutyric acid failed to inhibit it. Similar results have been reported with respect to lactobacilli by Waelsch, Owades, Miller & Borek (1946) and Borek, Miller, Scheiness & Waelsch (1946). We found the action of the sulphoxide on streptococci to be unaffected by cystine, methionine and homocystine. The relation of its action to glutamine thus showed a certain specificity. The compound was not markedly bactericidal (Table 8); quantitative details of its action on growth are given below.

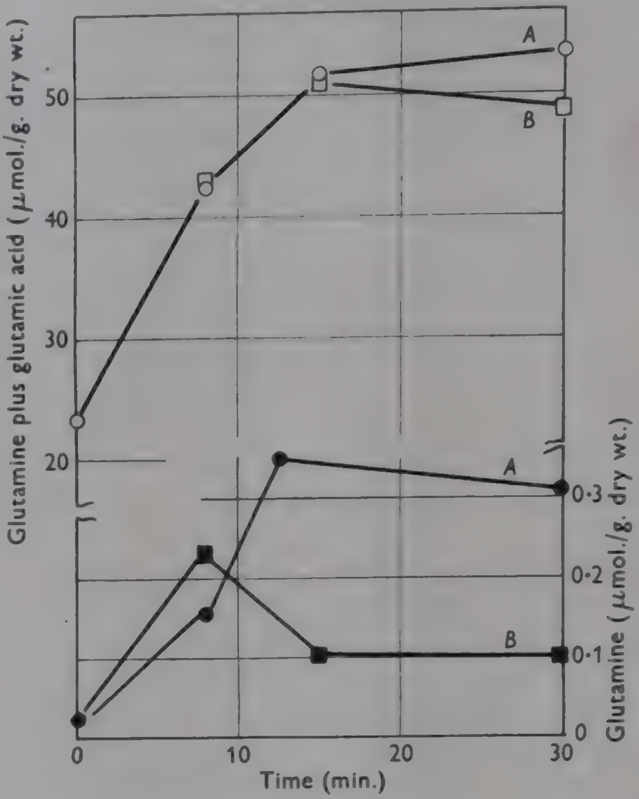

Fig. 9. Assimilation of glutamic acid and accumulation of synthesized glutamine in the presence of excess NH_3. Conditions as legend to Fig. 6, with: (*A*), 100 μmol. ammonium glutamate; and (*B*), 100 μmol. ammonium glutamate plus 500 μmol. NH_4Cl.

Inhibition of glutamine synthesis by the sulphoxide. Waelsch *et al.* (1946) suggested, on the basis of their growth experiments, that the sulphoxide affected lactobacilli through inhibiting their synthesis of glutamine. We have been able to demonstrate that the sulphoxide lowers the quantity of glutamine, formed from ammonium glutamate and glucose, and accumulating in streptococci under the experimental circumstances described in Table 13.

Many processes are occurring during such experiments, but the action of the sulphoxide was found to be a relatively specific one: the assimilation of glutamic acid is seen to be reduced to a smaller extent; in different experiments (Table 9) glycolysis was shown to be unaffected, and the breakdown of glutamine to glutamic acid not to be accelerated by the sulphoxide. It is likely, therefore, that the effect of the sulphoxide on the accumulation of glutamine is due to an inhibition of its synthesis.

Comparison of the effect of the sulphoxide on glutamine synthesis and on growth. The concentrations of methionine sulphoxide required for inhibition of growth were found to be comparable to those causing inhibition of the synthesis of glutamine. Both inhibitions depended also on the concentration of

Table 13. *Action of methionine sulphoxide on the accumulation of synthesized glutamine*

Organism	Reaction mixture* Ammonium glutamate (M)	Reaction mixture* Methionine sulphoxide (M)	Glutamic acid + glutamine of cells (µmol./g. dry wt.)	Glutamine of cells (µmol./g. dry wt.)	Glutamine accumulation in presence of sulphoxide as % of value in its absence $\left(\frac{c-a}{b-a}\right) \times 100$
Streptococcus faecalis, laboratory strain	0	0	52	0·30 (*a*)	—
	$2·8 \times 10^{-3}$	0	82	1·00 (*b*)	—
	$2·8 \times 10^{-3}$	0·028	76	0·65 (*c*)	50
	$0·55 \times 10^{-3}$	0	79	0·65 (*b*)	—
	$0·55 \times 10^{-3}$	0·028	73	0·40 (*c*)	28
Streptococcus faecalis, N.C.T.C. 6459	0	0	15·9	0·29 (*a*)	—
	3×10^{-3}	0	79	13·2 (*b*)	—
	3×10^{-3}	0·1	43	0·29 (*c*)	0
	3×10^{-3}	0·033	—	0·57 (*c*)	2

* The reaction mixture in all cases contained approximately the quantity of organisms, glucose, and phosphate which are quoted in Fig. 6.

glutamic acid which was present at the same time, and their comparison is therefore presented in the form given in Fig. 10. This shows that decrease in the rate of accumulation of synthesized glutamine to about half its normal value results in a partial inhibition of growth; this becomes nearly complete when the accumulation is reduced to about a quarter of its normal value. It is therefore feasible that the inhibition of growth is caused by inhibition of glutamine synthesis.

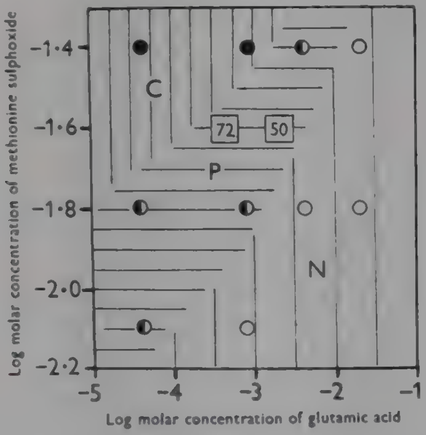

Fig. 10. Comparison of the degrees of inhibition caused in growth and in the accumulation of synthesized glutamine, by α-amino-γ-methylsulphinylbutyric acid. Manner of representation: see Fig. 5.

DISCUSSION

The course of glutamine metabolism

It has been shown that synthesis and breakdown of glutamine occur in streptococci in accordance with the reactions of Fig. 1. These reactions are indispensable to most streptococcal strains. Both in growing and in non-proliferating organisms they are associated with other processes such as glycolysis, and one reason for this lies in the requirement for a source of energy for the assimilation of glutamic acid and glutamine (Gale, 1947). The following considerations show, however, that this is not the only type of association between glutamine metabolism and glycolysis.

Some naturally-occurring streptococci do not synthesize glutamine, and in others the synthesis can be inhibited by α-amino-γ-methylsulphinylbutyric acid. In these cases added glutamine allows growth, by providing the product of the system which is missing or inhibited. This confirms our previous knowledge of the importance of the first reaction of Fig. 1. But need for the second reaction is not obviated by providing glutamic acid and NH_3. It is probable, therefore, that the second reaction is more complicated than indicated in Fig. 1 and other data support this view. First, glutamine stimulates streptococcal glycolysis. Secondly, the streptococcal system leading to production of NH_3 from glutamine may be contrasted with one of *Proteus morganii* (McIlwain, 1946*c*). This is easily extracted from the cells and does not require glucose for its activity. Glycolysis by *Pr. morganii* is unaffected by added glutamine.

Quantitative comparisons of processes involving glutamic acid in proliferating and non-proliferating bacteria

Four such comparisons, linking biological and chemical processes in streptococci, are made below on the basis of data from this and from other papers. The general problem which we wish to answer is whether the changes observed in non-proliferating cells are adequate in type and magnitude to form a basis for the behaviour of the organisms during growth.

Assimilation. The first problem is presented by the finding (Table 14) that streptococcal cells contain about 0·28–0·34 μmol./mg. of glutamic acid combined in the cell structure, together with a more variable quantity of some 0·01–0·1 μmol./mg. as free glutamic acid within the cell. The minimum rate of assimilation necessary to cause this accumulation can be calculated as follows. If $q=\mu$mol. of substance found per mg. of cells in a culture growing exponentially, and $Q=$ the instantaneous rate of assimilation of substance in μmol./generation time per mg., then by derivation such as that of Buchanan & Fulmer (1930), it can be shown that $q=Q\log_e 2$. The rates of assimilation necessary to account for the above cell contents of glutamic acid in cultures of a generation time of 20 min. are therefore

$$0{\cdot}6\ (=0{\cdot}29\times0{\cdot}70\times3)\ \text{to}\ 0{\cdot}9\ \mu\text{mol./mg./hr.}$$

Observed values for the rates of entry of glutamic acid and glutamine into non-proliferating streptococci have been collected in Table 14. They vary with the quantity of free glutamic acid and glutamine already present in the cells (see below), and the rates in cells of low free glutamic acid agree reasonably well with those calculated above as necessary for growth with generation time of 20 min. The process, in the form in which it has been observed in non-proliferating suspensions, is therefore adequate to provide the quantity of glutamic acid found in cells. There is not, however, a large margin between the quantity observed to enter, and that which is required to enter for the production of cells of the observed composition at the rate of one generation in each 20 min. Also, the cells do not appear capable of assimilating and storing quantities of glutamic acid or glutamine which are large in comparison with that combined in their substance; the rate of assimilation falls as cell content increases. Figs. 7, 8 and 9 show this (see also Gale, 1947). The quantity found as free acid has rarely been one-third that existing combined, presumably as protein. These observations lead to the expectation that inhibition of reactions concerned with streptococcal glutamic acid will be reflected relatively promptly in the generation time of

Table 14. *Rates of reactions which involve glutamic acid and glutamine, in growing and in non-growing streptococci*

Process	Organism	Rate at 37° (μmol./mg. dry wt./hr.)	Source of data; notes
Directly observed in non-proliferating suspensions, with glucose			
Assimilation of glutamic acid	*Faecalis*	0·40	Gale (1947); external glutamic acid, 9×10^{-3} M; initial streptococcal glutamic acid, 6 μmol./g.
Assimilation of glutamic acid	*Faecalis*	0·25	Fig. 6; external glutamic acid, 8×10^{-3} M; initial streptococcal glutamic acid, 25 μmol./g.
Assimilation of glutamine	*Faecalis*	0·72	Gale (1947); external glutamine, 9×10^{-3} M; initial streptococcal glutamic acid, 6 μmol./g.
Assimilation of glutamine	*Faecalis*	0·40	Figs. 6 and 7; external glutamine, 8×10^{-3} M; initial streptococcal glutamic acid, 25 μmol./g.
Conversion of glutamine to glutamic acid and NH_3	R	1·3–2·5	Table 4
Conversion of glutamine to glutamic acid and NH_3	*Faecalis*	0·5–0·6	Table 4
Accumulation of glutamine formed from ammonium glutamate	*Faecalis*	0·01	Fig. 8; organisms decomposed added glutamine at 0·6 μmol./mg./hr.
Rates calculated from content of cell or cell yield, as necessary to permit doubling of size of culture each 20 min.			
Assimilation of the glutamic acid which is found free in cells	*Faecalis*	0·02	Table 11; Gale (1947). Glutamic acid-poor cells of content 6–10 μmol./g.
Assimilation of the glutamic acid which is found free in cells	*Faecalis*	0·15	Table 13; Gale (1947). Glutamic acid-rich cells of content 50–100 μmol./g.
Assimilation of glutamyl residues of cell structure	*Faecalis*	0·60	Gale (1947); from cell content of 0·28 μmol./mg.
Assimilation of glutamyl residues of cell structure	—	0·66	Pollack & Lindner's (1942) estimate of cell content of glutamic acid (5 %)
Assimilation of minimum quantity of glutamine permitting growth of exacting streptococci	R	0·12	Roper & McIlwain (1948)

growing cultures, and instances of this are discussed below.

Glutamine synthesis. In the growth of non-exacting streptococci, the importance of the synthesis of glutamine has previously been indicated by indirect evidence, but the process itself has not been demonstrated. The accumulation of glutamine in cells provided with ammonium glutamate and glucose has now been found to be transitory only. If the maximum observed rate is expressed as a metabolic coefficient, it corresponds to a value of +0·01 μmol./mg./hr. As under such conditions glutamine is rapidly reconverted to ammonium glutamate, the rate of accumulation is unlikely to represent a rate of synthesis. Nevertheless, Table 12 shows that the rates of accumulation of glutamine in a series of streptococcal strains are inversely related to their need for added glutamine in growth. We may attempt to estimate the minimal needs of a streptococcus for glutamine (in distinction from glutamic acid) as follows. In an earlier paper (Roper & McIlwain, 1948) on the assay of glutamine, experimental conditions are chosen in which exacting streptococci are provided with excess ammonium glutamate but limited quantities only of glutamine, and are prevented from synthesizing glutamine from the ammonium salt. The quantity of organisms formed per unit weight of glutamine was found to be 15–20 mg. dry wt./μmol. of glutamine. 1 μmol. of glutamine plus glutamic acid would be contained in 2–3 mg. organisms of the glutamine plus glutamic acid content of those of Table 14. We may conclude that the exacting organisms require at least one-fifth of their glutamyl residues as glutamine, and if non-exacting organisms make similar use of glutamine, synthesis at the rate of about 0·12 μmol./mg./hr. would be required. This is much higher than the observed rate of accumulation.

Inhibition of glutamine accumulation by α-amino-γ-methylsulphinylbutyric acid. Although the observed rate of accumulation of glutamine is thus a fraction only of the probable rate of its synthesis, the sulphoxide affects the accumulation in a range of concentration closely paralleling that of its action on growth. Fig. 10 presents this well. We have also on occasions observed the sulphoxide to inhibit the assimilation of glutamic acid (Table 13), but at present express no opinion on possible causal relations between these effects.

Inhibition of glutamine breakdown. It appears from the similar comparison of Fig. 5 that inhibition of the conversion of glutamine to glutamic acid and NH_3 by the γ-hydrazide of glutamic acid also leads to a parallel inhibition of growth. This confirms the view, derived above, that this reaction in normal growth is associated with other changes, for glutamic acid and NH_3 do not prevent the action of the hydrazide on growth. Nevertheless, when the change, which in resting cells produces these substances, is inhibited, growth is also inhibited. Both the synthesis and breakdown of glutamine are thus of functional importance to streptococci.

SUMMARY

1. (*a*) The rate of glycolysis (per unit weight of organisms) by non-proliferating suspensions of β-haemolytic streptococci, which required glutamine for growth, fell on reducing the quantity of organisms in suspension below values of about 3 mg. dry wt./ml.

(*b*) Glutamine largely made good this deficiency, but sixteen substances related to glutamine in various ways were ineffective in doing this.

(*c*) During its action in glycolysis, ammonia was formed from glutamine, but related compounds added in place of glutamine (e.g. asparagine, glutaric acid amide or pyrrolidone-α-carboxylic acid amide) did not lead to ammonia formation.

(*d*) The system responsible for glutamine breakdown was not obtained in cell-free form, though arginine dihydrolase was so obtained.

2. (*a*) γ-Glutamylhydrazine inhibited the production of ammonia from glutamine during streptococcal glycolysis. The concentration of the hydrazide required for this effect increased with increase in the concentration of glutamine present; when this concentration was between $0{\cdot}4$ and $1{\cdot}6 \times 10^{-2}$M, the hydrazide at $0{\cdot}2$–2×10^{-2}M gave about 50% inhibition in the rate of ammonia formation.

(*b*) A similar range of concentrations of the reagents gave partial inhibition of growth, and again greater concentrations of the hydrazide were required in the presence of greater concentrations of glutamine.

(*c*) Many substances (including a number of phthaleins), which inhibited glycolysis, inhibited also the formation of ammonia from glutamine, and inhibited growth, but the relative sensitivities of these processes were different from those found in the case of the hydrazide, and supported the suggestion that the primary action of the hydrazide was in the breakdown of glutamine, and that this led to inhibition of growth.

3. (*a*) In the presence of glucose, glutamic acid or glutamine entered the cells of non-proliferating streptococci at rates of about 0·5–1 μmol./mg. dry wt./hr., which is adequate to account for the production of successive generations of cells of the observed glutamic acid content of 0·3–0·5 μmol./mg. and growing with a generation time of 20 min.

(*b*) Streptococcal cells contained a small quantity of material with the chemical and microbiological properties of glutamine. The quantity in streptococci which did not require added glutamine in growth could be increased by brief incubation with ammonium glutamate in the presence of glucose.

(*c*) Glucose also led to breakdown of glutamine, and the accumulation in streptococcal cells was found to be transitory only. This applied to glutamine added as such, as well as to that produced from ammonium glutamate and glucose. The maximum observed rate of accumulation of synthesized glutamine was about 0·01 μmol./mg./hr. in cells capable of decomposing added glutamine at the rate of 0·6 μmol./mg./hr. The rate was much lower, and probably zero, in organisms requiring added glutamine in growth.

(*d*) α-Amino-γ-methylsulphinylbutyric acid inhibited the accumulation of synthesized glutamine in non-exacting streptococci. In one strain, $2{\cdot}8 \times 10^{-2}$ M-solutions of the sulphoxide lowered the initial rate of accumulation from $2{\cdot}8 \times 10^{-3}$ M-ammonium glutamate by 50 %. These concentrations of reagents led to partial inhibition of growth of the organism, and the action of other concentrations on growth and metabolism were similarly correlated. The compound was without action on growth in the presence of glutamine and its action on growth in the presence of glutamate was presumably due to inhibition of glutamine synthesis. Both the synthesis and breakdown of glutamine are thus of functional value to the organisms.

We are greatly indebted to Miss E. Ellis for assistance during these investigations.

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The Estimation of the Leucine Isomers in Protein Hydrolysates by Infrared Analysis

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Certain of the monoamino-acid constituents of protein hydrolysates may be estimated by partition chromatography (Martin & Synge, 1941; Gordon, Martin & Synge, 1943; Tristram, 1946). This method, however, does not effect any separation of leucine $(CH_3)_2CH.CH_2.CH(NH_2)COOH$ and isoleucine $CH_3.CH_2.CH(CH_3).CH(NH_2)COOH$, the acetyl derivatives of which have identical properties under the conditions of the Martin & Synge technique. The present investigations were directed towards obtaining a rapid and accurate spectroscopic method for analyzing the isoleucine/leucine ratio in the leucine fractions separated from protein hydrolysates by partition chromatography.

The possibility of analyzing a mixture of leucine and isoleucine by means of differences in their vibration spectra was first suggested by Edsall (1938) in work on the Raman spectra of amino-acids, but, as Edsall noted, the Raman technique presents many difficulties in such a problem. In the course of spectroscopic work on amino-acids, peptides and proteins, we recorded the infrared spectra of leucine and isoleucine and their acetyl derivatives, the latter for the first time. As might be anticipated from the differences in the hydrocarbon skeletons of such isomers, marked differences were found between the spectra of leucine and isoleucine, and between those of their acetyl derivatives. The spectra of the latter are illustrated in Fig. 1, from which it is clear that the 714 cm.$^{-1}$ band of acetyl-L-leucine and the 736 cm.$^{-1}$ band of acetyl-L-isoleucine should provide a ready means of distinguishing these two isomers and estimating their relative abundance in a mixture. Using partition chromatography (Gordon *et al.* 1943; Tristram, 1946) it is possible to isolate a representative mixture of the leucine isomers as their acetyl derivatives. For this reason, the infrared analyses have been carried out on the acetamido acids rather than on the free amino-acids.

The applicability of an infrared analysis was first tested on a number of synthetic mixtures of acetyl-L-leucine and acetyl-L-isoleucine. The method was then extended to the examination of the leucine isomers obtained from various control mixtures, and finally leucine fractions from the hydrolysates of various proteins were studied.

ANALYTICAL PROCEDURE

Chemical

The leucine isomers were isolated by the method of Martin & Synge (1941). In the early stages of this work the procedure was to isolate the isomers on the quantitative column, using *c.* 25 mg. of protein. For a total leucine content of 10% this entailed the working up of some eight fractions. Now the R_F values of acetyl leucine and acetyl isoleucine are identical so that as long as the isolated samples are not submitted to recrystallization there is no need to attempt their quantitative isolation. Accordingly, the later samples were prepared qualitatively using 100–200 mg. of protein, the aim being to obtain about 20 mg. of mixed acetamido acids.

Procedure. The hydrolysate, equivalent to 100–200 mg. of protein, was acetylated in the usual manner (Gordon *et al.* 1943) using *c.* 20 ml. 6N-NaOH and 5·0 ml. acetic anhydride. After acidification to pH 1 with 10N-H_2SO_4, the mixture was adsorbed on silica gel, transferred to a large column (4·0 cm. diameter) and extracted with 1 l. chloroform-17% butanol (Tristram, 1946). The acetamido acids were then prepared for analysis and the leucine isomers isolated by the procedure outlined by Tristram (1946). In order to eliminate any acetylphenylalanine and acetylvaline, only the centre portion of the leucine band was collected. It was freed from colouring matter and indicator by passing it through water-saturated silica gel using *cyclo*hexane-5% propanol. The solvent was then removed and the acetamido acids allowed to crystallize in a vacuum desiccator.

Physical

Preparation of specimens for analysis. For accurate analytical work using infrared spectra, the sample should be either in the gaseous or liquid state, although solids which can be obtained as thin plates or films of which the thicknesses can be accurately determined are acceptable. Solvents having no intense absorption over the spectral regions investigated are very scarce in infrared work, and none of these was suitable for this problem. Estimations were accordingly made on the solid material suspended as a paste in liquid paraffin (Nujol). The acetamido acids were finely ground in a mortar and well mixed with an approximately equal quantity of Nujol until a smooth homogeneous paste was obtained. This paste was then pressed into a thin layer (approx. 30 μ) between two rocksalt plates and placed in the spectrometer beam. With this technique, the total amount of acetamido acids suspended in the radiation beam is not accurately known. The method accordingly leads to estimates of the relative rather than the absolute concentrations of the two isomers.

Quantities of materials used. The amount of material used in the analyses was limited by the yield of leucines obtained from the chromatographic separation; experiments were originally made on about 13–15 mg. of material, but later, smaller quantities, *c.* 7–10 mg., were found to be satisfactory for an assay if the sample was well dried and crystalline. The economy was principally achieved by having a suspension layer of very small area, placed close to the entrance slit of the spectrometer; a further reduction in quantity could probably be obtained by bringing the incident beam of the spectrometer to a subsidiary focus of small area. The concentration of the acetamido acid in the suspensions was such that the relevant absorptions were near the optimal percentage for accurate estimation.

Spectroscopic details. The earlier experimental work was done on a Hilger D. 88 spectrometer equipped with a rocksalt prism. The later work, on which all the data in this paper are based, was performed on a double-beam spectrometer (Hilger D. 209) with automatic pen recording (Sutherland & Thompson, 1945). At 700 cm.$^{-1}$, the rocksalt prism begins to absorb radiation, and the small amount of energy available makes accurate quantitative work difficult; accordingly we used a potassium bromide prism, which transmits to much lower frequencies in the infrared.

The resolving power of the D. 88 spectrometer is comparable with that of the D. 209 spectrometer at 700 cm.$^{-1}$ when the latter is equipped with a KBr prism. Recording of both spectrometers was automatic, and the spectral range 760–700 cm.$^{-1}$ was scanned several times for each specimen, to check reproducibility. In this way errors in the shape of the bands and the exact height and location of the maxima could be minimized.

Choice of bands for analysis. The infrared absorption spectra of acetyl-L-leucine, acetyl-L-isoleucine, acetyl-DL-leucine and acetyl-DL-isoleucine are shown in Fig. 1, in the region 1800–650 cm.$^{-1}$. The absorption bands are listed in Table 1. Although the spectra of the laevo compounds differ in many respects, it is advisable to choose two bands for analytical work which lie fairly close together. This is because these bands can then be examined under identical spectroscopic conditions (e.g. slit width).

It is clear from the spectra (Fig. 1) that the band of acetyl-L-leucine at 714 cm.$^{-1}$ and that of acetyl-L-isoleucine at 736 cm.$^{-1}$ satisfy this condition, and are yet sufficiently separated to be well resolved by the spectrometer. These bands do in fact overlap to a slight extent so that the intensities of absorption at 736 and 714 cm.$^{-1}$ are not entirely due to acetyl-L-isoleucine and acetyl-L-leucine respectively. However, this can be taken into account in the calculations.

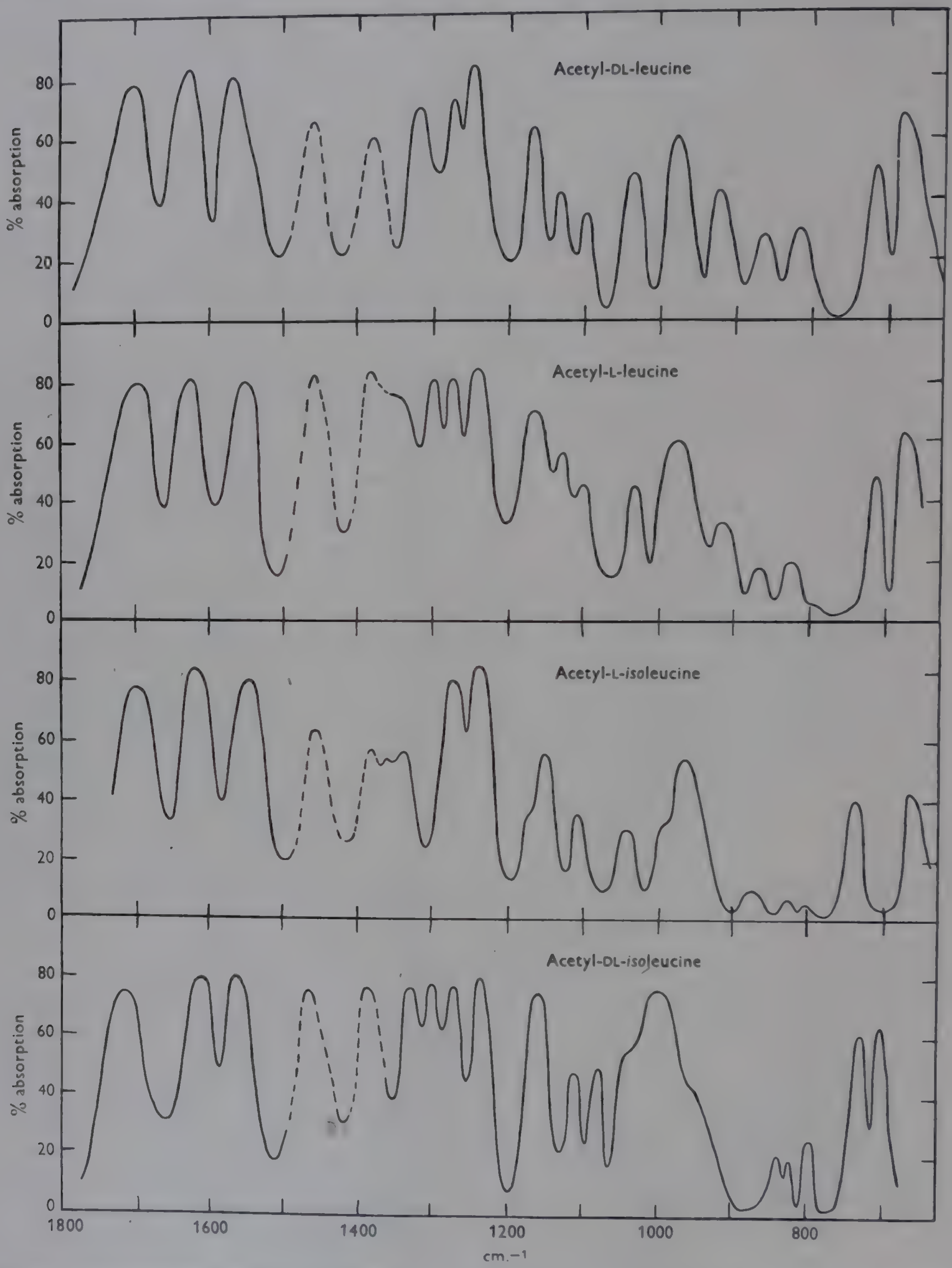

Fig. 1. Infrared absorption spectra of acetylleucines, 1800–650 cm.$^{-1}$. (Broken lines indicate Nujol absorption.)

The effects of racemization. The estimations were made on the assumption that the acetamido acids were present entirely in the laevo configuration and that no appreciable amount of racemization had occurred. The absorption spectra of the DL-acetamido acids are seen (Fig. 1 and Table 1) to be somewhat different from those of the corresponding laevo compounds. This effect had been shown earlier by Wright (1937, 1939) for many amino-acids. The presence of an appreciable proportion of racemized material in the chromatographic fraction may therefore render the estimation inaccurate. Acetyl-DL-leucine has an absorption peak at 714 cm.$^{-1}$ with approximately the same extinction coefficient as that of acetyl-L-leucine, but acetyl-DL-isoleucine gives rise to a broad absorption band centred at 726 cm.$^{-1}$ which would contribute to the absorptions measured at 736 and 714 cm.$^{-1}$. The spectra of corresponding L- and DL-leucines are not sufficiently different in the region 3600–750 cm.$^{-1}$ to enable a convenient check to be made on the presence of low proportions of DL-isomers in the fractions, but it is believed that the proportion of racemates in the specimens assayed is quite low.

Table 1. *Absorption maxima of the acetylleucines* 1800–650 *cm.*$^{-1}$

Acetyl-L-leucine	Acetyl-DL-leucine	Acetyl-L-isoleucine	Acetyl-DL-isoleucine
1695	1703	1703	1720
1624	1625	1622	1608
1554	1567	1547	1566
(1460) Nujol	(1460) Nujol	(1460) Nujol	(1460) Nujol
(1380) Nujol	(1380) Nujol	(1380) Nujol	(1380) Nujol
1347	1316	1364	1329
1298	1271	1339	1298
1276	1247	1270	1270
1244	1166	1238	1232
1164	1131	1170	1157
1127	1099	1150	1108
1103	1035	1105	1072
1034	976	1043	1042
974	920	993	996
917	864	965	835
863	815	876	820
822	714	823	793
796	681	803	726
714	—	736	701
678	—	665	—

Corrections for spurious and scattered radiation. It is important in quantitative analytical work in the infrared region to make allowance for spurious radiation. This is usually of higher frequency than the spectral range under examination, and may therefore have an appreciable magnitude in comparison with the energy being estimated. This is particularly important in the present case where the spectral region 750–700 cm.$^{-1}$ is so far removed from the region of maximum energy of the Nernst source. Spurious radiation of frequency greater than about 900 cm.$^{-1}$ is allowed for by placing a fluorite plate in the spectrometer beam to measure the effective zero energy at 750–700 cm.$^{-1}$ (fluorite is opaque for frequencies less than 900 cm.$^{-1}$). This zero setting was found to be appreciably different from that obtained when the beam was cut off with a metal shutter, showing that a small proportion of high-frequency radiation greater than 900 cm.$^{-1}$ was being received by the detector when the 750–700 cm.$^{-1}$ region was being scanned. That the amount of radiation scattered by the solid particles in suspension is probably very small in this region of the spectrum has been shown by comparing the degree of transmission of the sample, at a frequency well removed from an absorption band, with the degree of transmission of a layer of Nujol between rocksalt plates at that same frequency. Scattering effects have therefore been neglected.

Methods of calculating concentrations

We assume the validity of Beer's law, viz.

$$I_\nu = I_{0\nu} e^{-kc}, \tag{1 a}$$

relating the intensity of the transmitted radiation I_ν, the incident intensity $I_{0\nu}$ of the same frequency, the number of gram molecules c placed in the spectrometer beam and the molecular extinction coefficient k. We further assume that Beer's law may be extended additively for mixtures of two components,

$$I_\nu = I_{0\nu}\, e^{-(kc+k'c')}. \tag{1 b}$$

The incident intensity $I_{0\nu}$ is measured experimentally as the intensity of radiation of frequency ν, transmitted through the absorption cell containing Nujol only. This procedure allows for radiation reflected from the cell faces, and radiation absorbed and scattered by the cell and the Nujol.

Let c = number of gram molecules of acetyl-L-leucine, c' = number of gram molecules of acetyl-L-isoleucine, I_{01} = the incident intensity at 714 cm.$^{-1}$, I_1 = transmitted intensity at 714 cm.$^{-1}$, I_{02} = incident intensity at 736 cm.$^{-1}$, I_2 = transmitted intensity at 736 cm.$^{-1}$, k_1 = molecular extinction coefficient of acetyl-L-leucine at 714 cm.$^{-1}$, k_2 = molecular extinction coefficient of acetyl-L-leucine at 736 cm.$^{-1}$, k_1' = molecular extinction coefficient of acetyl-L-isoleucine at 714 cm.$^{-1}$, k_2' = molecular extinction coefficient of acetyl-L-isoleucine at 736 cm.$^{-1}$. Then, from Beer's law,

$$\log_e \frac{I_{01}}{I_1} = k_1c + k_1'c', \tag{2 a}$$

$$\log_e \frac{I_{02}}{I_2} = k_2c + k_2'c'. \tag{2 b}$$

Let d_1 = optical density at 714 cm.$^{-1}$ $= \log_e \dfrac{I_{01}}{I_1}$, (3 a)

and d_2 = optical density at 736 cm.$^{-1}$ $= \log_e \dfrac{I_{02}}{I_2}$. (3 b)

Then, solving (2 a) and (2 b) for c and c', we have

$$c = \frac{d_1k_2' - d_2k_1'}{k_1k_2' - k_2k_1'}, \tag{4 a}$$

$$c' = \frac{d_1k_2 - d_2k_1}{k_2k_1' - k_1k_2'}, \tag{4 b}$$

or

$$\frac{c'}{c} = \frac{d_1k_2 - d_2k_1}{d_2k_1' - d_1k_2'}. \tag{5}$$

The terms of the right-hand side of equation (5) are homogeneous in d, so that common logarithms may be used throughout. We may rewrite this

$$\frac{c'}{c} = K\frac{d_2 - d_1k_a}{d_1 - d_2k_b}, \tag{6}$$

where K, k_a, k_b, are constants related to the molecular extinction coefficients, and which are easily determined from the experimental data, i.e.

$$K=\frac{k_1}{k_2'},\quad k_a=\frac{k_2}{k_1},\quad k_b=\frac{k_1'}{k_2'}.$$

This equation enables the ratio of the quantities of the two isomers to be calculated in a manner independent of the total quantity of material present.

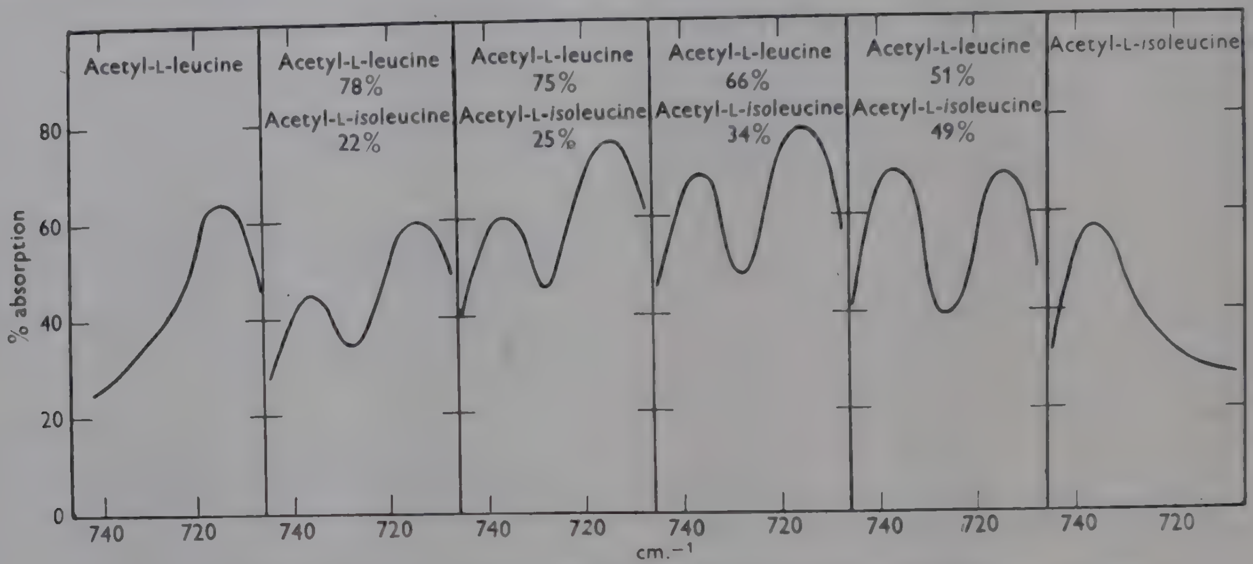

Fig. 2. Absorption spectra of some mixtures of known composition of acetyl-L-leucine and acetyl-L-isoleucine, 745–710 cm.$^{-1}$.

Evaluation of K, k_a, k_b. k_a, k_b are evaluated from the absorption curves for pure acetyl-L-leucine and acetyl-L-isoleucine. For the pure leucine isomer, $c'=0$, and from equation (6), $k_a=\left[\frac{d_2}{d_1}\right]_0$, the subscript 0 indicating that d_1 and d_2 are the optical densities for the pure leucine isomer.

Similarly, for the pure isoleucine isomer, $k_b=\left[\frac{d_1}{d_2}\right]_0'$.

If a series of isomeric mixtures of known proportions is examined and the function $\frac{d_2-d_1k_a}{d_1-d_2k_b}$ plotted graphically against c'/c, a straight line should be obtained, the value of the slope being the constant K. Having determined the line and relation between c'/c and the function of the optical densities d_1 and d_2, the value of c'/c may be obtained directly from the graph for the value of

$$(d_2-d_1k_a)/(d_1-d_2k_b)$$

given by any mixture of unknown composition.

RESULTS

The calibration data

Synthetic mixtures of the pure crystalline acetyl-L-leucines were examined over a range varying from 0 to 50% isoleucine; mixtures containing more than 50% isoleucine were not examined, analytical data for the proteins indicating that isoleucine is invariably found in smaller amounts than leucine. Each synthetic mixture was examined at different path lengths (giving different absorptions at the maxima), and the conditions studied under which the assumptions of Beer's law stated in the previous section, are justified. The relevant absorption bands of some of these mixtures are shown in Fig. 2.

It was found that, for a given mixture, the value of $(d_2-d_1k_a)/(d_1-d_2k_a)$ is constant, within the limits of experimental error, for a moderate range of d_1 and d_2. For path lengths giving greater than about 85% absorption, the intensity of absorption of the weaker band is apparently enhanced, giving high values of c'/c. When the bands are weak (the stronger band being less than about 40% absorption), the intensities cannot be measured with sufficient precision to enable c'/c to be determined accurately. We have therefore examined samples such that the absorption intensities of the stronger band lie between about 40 and 80%, both in the calibration and in the estimation of mixtures of unknown composition.

The evaluation of the calibration data is set out in Table 2 (*a*). Substituting the values $k_a=0{\cdot}36$ and $k_b=0{\cdot}40$, from this table, into equation (6),

$$c'/c=K(d_2-0{\cdot}36d_1)/(d_1-0{\cdot}40d_2). \qquad (7)$$

But $d_1=\log_{10}100/I_1$ and $d_2=\log_{10}100/I_2$, so that

$$c'/c=K\,(1{\cdot}28-\log_{10}I_2+0{\cdot}36\log_{10}I_1)/(1{\cdot}20-\log_{10}I_1+0{\cdot}40\log_{10}I_2). \qquad (7a)$$

Equation (7*a*) is the form of equation (7) which is used in the numerical calculations.

The absorption data for mixtures of known composition are given in Table 2 (*b*); the values of $(d_2-0{\cdot}36d_1)/(d_1-0{\cdot}40d_2)$ calculated from these give a straight line when plotted against the corresponding values of c'/c (Fig. 3); this is taken as the working curve in the analysis of unknown mixtures. When $c'=0$ (pure leucine), $(d_2-0{\cdot}36d_1)/(d_1-0{\cdot}40d_2)$ is equal to zero, so that the curve passes through the origin.

Table 2. *Calibration data*

(*a*) Evaluation of k_a and k_b

	Percentage transmission at 714 cm.$^{-1}$ (I_1)	Percentage transmission at 736 cm.$^{-1}$ (I_2)	k_a	k_b
Acetyl-L-leucine	38·5	71·0	0·358	—
	36·0	69·5	0·357	—
Acetyl-L-isoleucine	71·0	43·0	—	0·406
	75·0	48·5	—	0·398

Mean value of $k_b=0{\cdot}36$. Mean value of $k_b=0{\cdot}40$.

(*b*) Transmission of synthetic mixtures

Percentage ratios leucine ; isoleucine	c'/c	Transmissions I_1	Transmissions I_2	$\frac{d_2-0{\cdot}36d_1}{d_1-0{\cdot}40d_2}$	Mean value
51 : 49	0·96	31·5	30·5	1·14	1·15
		56·5	55·0	1·18	
		60·5	59·5	1·15	
66 : 34	0·515	19·0	28·0	0·59	0·59
		20·5	29·5	0·59	
		21·5	31·0	0·58	
		42·0	52·5	0·56	
75 : 25	0·33	24·0	39·0	0·41	0·40
		61·5	73·0	0·39	
		66·0	78·0	(0·31)	
78 : 22	0·28	40·0	55·0	0·39	0·38
		61·0	73·0	0·36	
		60·0	71·5	0·40	

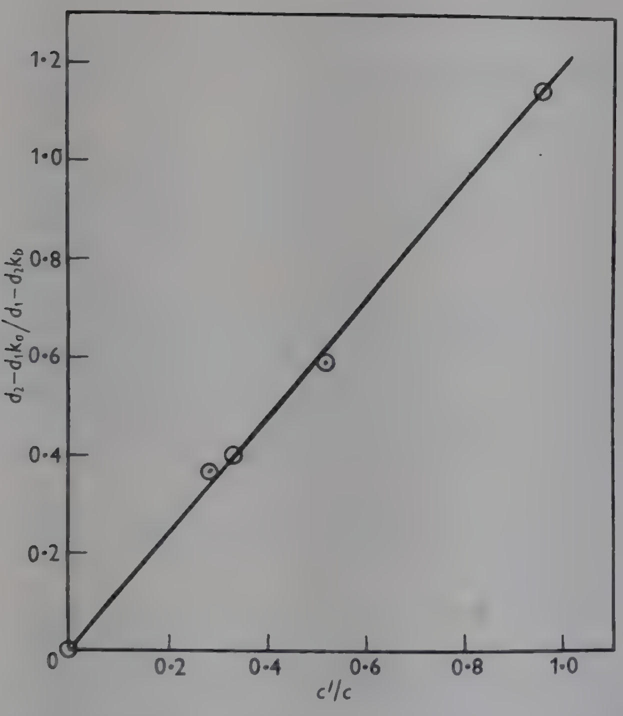

Fig. 3. Calibration curve for leucine-isoleucine analysis.

The calibration data in Table 2 are given in detail to enable an estimate to be made of the 'spread' of the results obtained by this method of analysis.

Control mixtures

Two control experiments were performed to test the validity of the chemical procedure.

(i) Known proportions of the acetyl-L-leucines were evaporated together from solution in the same way as the leucine fractions from the chromatograph column. The mixture had not been treated chromatographically. The results of the spectroscopic analysis are shown in Table 3. The agreement between the analytical values and the original composition indicates that the spectra of the isomers after these have been crystallized together from solution are still additive.

Table 3. *Estimation of isoleucine in a control mixture of leucines*

Isoleucine as % total leucine isomers: Present in original mixture	Isoleucine as % total leucine isomers: Estimated spectroscopically
32·2	30·3

(ii) A mixture of acetamido acids, in the proportions in which they occur in an insulin analysis (Tristram, 1946), and having an isoleucine/leucine ratio of 1 : 4, was passed through the chromatograph column. The leucine fraction was isolated and the isomer ratio estimated spectroscopically (Table 4).

Table 4. *Control experiment on 'synthetic insulin hydrolysate'*

Isoleucine as % total leucine isomers: Present in original 'hydrolysate'	Isoleucine as % total leucine isomers: Estimated spectroscopically
19·8	18·7, 18·7, 17·5

The estimated ratios agree within 2 % with the ratio originally present, the proportion of isoleucine estimated being low. This control experiment indicates that the leucine isomers are not differentiated in the chromatograph column.

Proteins

The isoleucine/leucine ratio has been estimated in the hydrolysates of three proteins, casein, edestin and insulin (two samples from independent hydrolysates). The ratios are given in Table 5. Several assays were made in each sample at varying optical densities; consistent values of the isoleucine/leucine ratio were obtained from assays having absorptions near the optimal value for accurate measurements. The spectra of these samples were examined carefully in the range 1800–700 cm.$^{-1}$ and compared with those of similar synthetic mixtures of acetyl leucine and isoleucine. Agreement throughout the spectral range was good and no evidence of extraneous bands, arising from impurities, was found. The spectra of acetyl-L-valine, and the acetyl-DL-leucines were particularly examined in this connexion.

Table 5. *The isoleucine/leucine ratio as determined spectroscopically in some proteins*

Protein	c'/c	Isoleucine as % of total leucines	
Casein	0·46	32	
Edestin	0·24	19	Two assays
	0·20	17	
Insulin (first sample)	0·205	17	Two assays
	0·19	16	
Insulin (second sample)	0·23	18·5	Three assays (mean value 15–18 %)
	0·17	14·5	
	0·18	15·3	

DISCUSSION

The reliability and accuracy of the method

The assumption made in these calculations is that the relative absorption intensities of the isomers at two fixed frequencies can be expressed by Beer's law, in a form which is independent of the total amount of absorbing material. The two isomers are examined together as a physical mixture of the solids. The possibility that intermolecular interaction (such as might arise by crystallizing the isomers together) could invalidate the use of Beer's law has been shown to be absent. The validity of these assumptions is confirmed by the experimental data from the synthetic mixtures (Fig. 3), which show a constant value for K, the slope of the curve.

Reproducibility of the percentage absorptions which are measured is checked by scanning each mixture twice or more; the percentage absorptions are then considered accurate to $\pm 0{\cdot}5\,\%$ of absorption. The corresponding error in the percentage of isoleucine in the mixture is plotted in Fig. 4 against the percentage of isoleucine. It is thus seen, for example, that when the percentage of isoleucine is near 19 %, this figure is expressed as $(19 \pm 1{\cdot}5)\,\%$. In order not to overstate our accuracy, the errors in measurement of the two bands have been assumed to be cumulative. The independence of total concentration is ascertained by examining each sample at two or more optical densities. Reasonably good agreement is obtained when the absorption of the stronger component is within the range 40–80 %, and when the weaker band is not too feeble to be measured accurately.

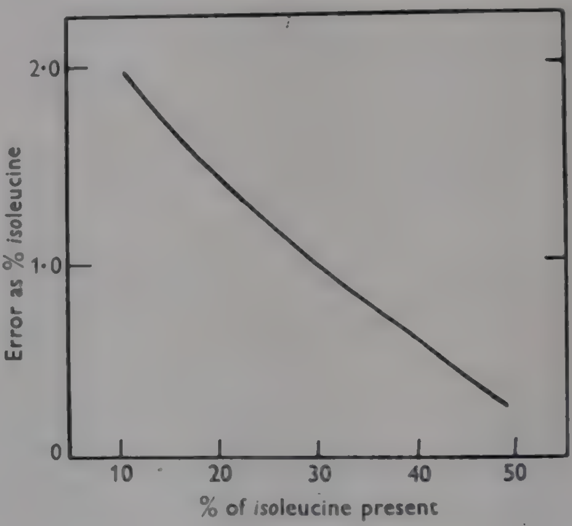

Fig. 4. Error curve for isoleucine estimation, assuming errors of $\pm 0{\cdot}5\,\%$ in measuring absorption bands.

The method is capable of refinements and by adopting a standard procedure of working, with adequate controls, it can probably be made a very reliable method of amino-acid analysis. Results are only reproducible if identical spectroscopic working conditions both for the calibration and assays are maintained.

As discussed earlier, racemization would cause errors in the present spectroscopic estimations of the isoleucine/leucine ratio.

Whether or not the leucine fraction is fully recovered from the chromatograph column, the spectroscopic method of analysis should yield a reliable value of the isoleucine/leucine ratio.

Comparison of the method with microbiological assay

The greatest difference between the spectroscopic method and that of microbiological assay is the relatively large amount of material needed for the former. For a spectroscopic analysis, we require at present 5–10 mg., compared with the few μg. required for a microbiological assay. Moreover, the spectroscopic method, as used at present, gives the relative concentrations of the isomers, whereas absolute concentrations of the amino-acids are given by microbiological assay.

The advantage of the spectroscopic method over microbiological assay is that a highly characteristic physical property of the molecule (its vibration spectrum) is being used. The microbiological method is subject to doubts, particularly regarding lack of specificity of the organism, and interference of other constituents in a mixture. The microbiological method is rendered inaccurate by racemization, the D-form in general not being estimated (see Dunn, Camien, Shankman & Block, 1947), and the assay therefore being low in such a case. The effect of racemization in spectroscopic estimation has already been discussed, but there is little doubt that a more refined technique could make due allowance for this. We would stress that the present investigations have been of an exploratory nature, and that the method is capable of greater accuracy with a carefully standardized routine.

Discussion of analysis

The mean values of percentages of isoleucine in total leucine fractions shown in Table 5 are considered reliable to ± 1·5 % (see Fig. 4), although the control experiments suggest that there may be a tendency for the present results to be systematically low. The comparison with microbiological values is given in Table 6. It will be observed that the spectroscopic values for casein and insulin are close to those previously obtained by microbiological assay, but the isoleucine proportion is slightly low in both cases.

Table 6. *Comparison of spectroscopic values of the isoleucine/leucine distribution with microbiological values*

	Percentage of isoleucine in total leucine fraction	
	Spectroscopic	Microbiological assay
Casein	32	38·5[*], 39·5[†], 39·2[‡], 36·2[§]
Edestin	18	45·5[‖]
Insulin	15–18	17·8[¶]

[*] Hier, Graham, Friedes & Klein (1945).

[†] Kuiken, Norman, Lyman, Hale & Blotter (1943).

[‡] Dunn, Camien, Rockland, Shankman & Goldberg (1944).

[§] Stokes, Gunness, Dwyer & Caswell (1945).

[‖] Smith, Greene & Bartner (1946); Smith & Greene (1947).

[¶] Brand (1946).

An earlier spectroscopic value for the ratio of the leucine isomers, which was given in a publication by Tristram (1946), was in error, due to imperfect calibration data obtained in the preliminary stages of the investigations on insulin.

The value of comparing one method with another based upon different principles is indicated very strikingly in the case of the ratio of the leucine isomers of edestin. Smith *et al.* (1946) and Smith & Greene (1947) reported that the leucine (*Lactobacillus arabinosus*) and isoleucine (*Streptococcus faecalis*) contents of edestin were 4·25 and 3·55 % respectively (i.e. 54·5 % of leucine). Infrared analysis suggests that the distribution is nearer 80 % leucine (Table 5).

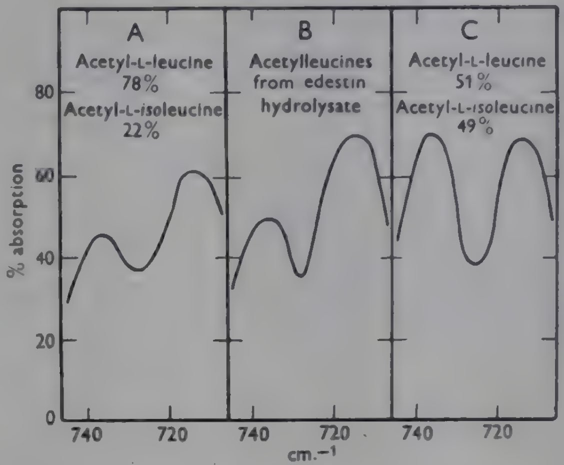

Fig. 5. Comparison of spectra of acetylleucines from edestin hydrolysate with mixtures of known composition.

It has been shown that the chromatographic separation of the acetylleucines fraction does not effect any concentration of isoleucine, and whatever the recovery of the total leucine isomers, the ratio is unaltered. Two of the calibration mixtures (Table 2*b*), containing respectively 51 and 78 % leucine, are near to the distributions suggested respectively by microbiological and spectroscopic methods, and the absorption curves of these mixtures are compared in Fig. 5 with that obtained from the leucines fraction of edestin.

SUMMARY

1. The infrared absorption spectra of leucine and isoleucine and of their acetyl derivatives have been examined in the range 1800–650 cm.$^{-1}$. The spectra of each pair of isomers show marked differences which make it easy to distinguish the isomers.

2. A spectroscopic method of analyzing isoleucine/leucine mixtures has been investigated (using as indicators characteristic bands near 700 cm.$^{-1}$ of the acetyl derivatives) and tested on synthetic mixtures of the L-isomers.

3. The applicability of the spectroscopic method of analysis for estimating the isoleucine/leucine ratio in the leucine fraction of protein hydrolysates has been tested by control experiments on mixtures of leucine and isoleucine subjected to the treatment employed in protein analyses.

4. The study of the method in the determination of the isoleucine/leucine ratio in the leucine fraction from the hydrolysates of casein, insulin and edestin gave results which agree with those obtained by microbiological methods except in the case of edestin where there is a very large discrepancy.

We are grateful to Prof. A. C. Chibnall, at whose instigation this investigation was undertaken, for his interest and helpful advice. We are indebted to the Medical Research Council (S.E.D.) and to the Agricultural Research Council (G.R.T.) for grants during the tenures of which this investigation was undertaken.

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Studies on Vitamin A

5. THE PREPARATION OF RETINENE$_1$—VITAMIN A ALDEHYDE

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The state of knowledge concerning photoreception and the role of rhodopsin (visual purple) has been surveyed recently (Granit, 1947) so that only a brief introduction is necessary here.

Wald (1935–6) found that dark-adapted retinas, which are pinkish purple in colour owing to the presence of relatively large amounts of rhodopsin, turn yellow on illumination. Light petroleum will extract from retinas exposed to light a material which Wald called *retinene*. The petroleum solution, freed from solid matter by centrifuging, shows an ultraviolet-absorption maximum near 365 mμ., displaced to 385 mμ. when chloroform is the solvent. When the antimony trichloride reagent, used in vitamin A studies, is added to a chloroform solution of retinene, a blue colour is produced. The blue solution exhibits a well-defined absorption band with a maximum at 664 mμ. Wald postulated for scotopic vision a cyclic process into which entered vitamin A, retinene and visual purple.

In later studies, Wald (1937, 1938–9 *a*, *b*) found that a similar extraction procedure applied to retinas of fresh-water fish, in which rhodopsin is largely replaced by porphyropsin (visual violet), yielded retinene$_2$. This substance is similar to retinene$_1$ but its absorption spectrum is slightly displaced in the direction of longer wave-lengths, as also is the characteristic absorption maximum of the colour test. The discovery that vitamin A$_2$ (Edisbury, Morton & Simpkins, 1937; Lederer & Rosanova, 1937) tends to take the place of vitamin A in fresh-water fish as compared with sea fish and mammals indicated that retinene$_2$ bears the same relationship to vitamin A$_2$ as retinene$_1$ to vitamin A. This is not surprising, as there was already ample evidence of a connexion between vitamin A deficiency and night blindness associated with retarded formation of rhodopsin (visual purple) in the dark-adaptation process.

The discovery of the retinenes was a notable advance, but paucity of material has delayed its exploitation. The amounts obtainable from eyes are too minute to permit orthodox constitutional investigations and for nearly a decade the substances have remained known only by qualitative spectroscopic characteristics quoted in Table 1.

Table 1. *Absorption maxima of substances concerned in visual processes*

Substance	$\lambda_{\max.}$ (mμ.)	Solvent	$SbCl_3$ colour test (in chloroform) $\lambda_{\max.}$ (mμ.)
Vitamin A	326	*cyclo*Hexane	617 inflexion 582
Vitamin A$_2$	345–350	*cyclo*Hexane	693
Rhodopsin (visual purple)	502	1% aqueous digitonin	
Porphyropsin	520–530		
Retinene$_1$	369·5	Light petroleum	664
	389	Chloroform	
Retinene$_2$	390	Petrol	>705
	405	Chloroform	

The conclusion drawn by Wald, that retinene$_1$ is a carotenoid and rhodopsin a conjugated protein with retinene as its prosthetic group, has been much quoted, but it is rather insecurely based. Some writers use the term 'carotenoid' as a class designation for highly coloured C_{40} polyenes including those containing hydroxyl and carboxyl groups. Others include in the class C_{20} polyenes such as vitamin A, which are almost colourless. It is arguable that this loose use of the word carotenoid is permissible, but the suggestion that retinene is itself the prosthetic grouping of visual purple is difficult to reconcile with the displacement of the 369 mμ. maximum of retinene to 502 mμ. in visual purple.

No questions of terminology or tentative interpretation can, however, weaken Wald's demonstration that the vitamins A and the visual pigments are somehow linked through the retinenes. From a chemical standpoint, the preparation of the pure retinenes, the elucidation of their structures, and the changes they can be made to undergo are major objectives in the study of visual processes.

Morton (1944), in a survey of the problem based primarily on the interpretation of absorption spectra, came to the conclusion that retinene$_1$ must be the aldehyde of vitamin A. By far the most plausible explanation of the 369 mμ. absorption maximum of retinene$_1$ was the replacement of CH_2OH in vitamin A by CHO, as this would give six conjugated double bonds instead of five, and so account for the displacement from 326 to 369 mμ. Oxidation at any other point in the vitamin A molecule could not fail to displace the absorption in the opposite direction. Similar arguments were valid for the retinene$_2$-vitamin A_2 system. The task of preparing such an aldehyde seemed formidable because of the five conjugated double bonds present in the vitamin A molecule. It was not easy to see how ordinary oxidative processes could be restricted to the terminal CH_2OH group and a study of the literature showed that in the Oppenauer reaction any aldehyde formed would tend to undergo further change. Considerations of that kind did not, however, dispose of the matter.

Direct oxidation of vitamin A might be expected to result in simultaneous attack upon the terminal CH_2OH group and upon the double bonds, yielding a variety of products, but the possibility that a little vitamin A aldehyde might be formed, which would escape further attack, seemed worthy of investigation, especially as chromatographic adsorption gave some promise of a separation of the mixture. Morton & Goodwin (1944), by shaking vitamin A concentrates dissolved in light petroleum with dilute aqueous $KMnO_4$ in the presence of a little H_2SO_4 and chromatographic separation of the products, always obtained some fractions which showed an absorption band at 365–370 mμ. in saturated hydrocarbon solvents and at 385 mμ. in chloroform. In the latter solvent the solute gave a 664 mμ. maximum with the antimony trichloride reagent. The yields of spectroscopically homogeneous material were small, but there could be no doubt that retinene was an oxidation product of vitamin A, and that it was very probably the simple aldehyde.

The suggestion that retinene was vitamin A aldehyde was soon confirmed by Hunter & Hawkins (1944), who obtained from vitamin A by the Oppenauer reaction a material showing two absorption maxima at 350 and 368 mμ. respectively, and giving a colour-test maximum at 657 mμ. It also yielded a 2:4-dinitrophenylhydrazone, m.p. 207–209°, and was converted to vitamin A by the Pondorff reduction. Hunter & Hawkins were kind enough to allow two of us to test their product. The difference between 657 and 664 mμ. turned out to be a matter of spectroscopic technique only, but the presence of an ultraviolet maximum at 350 mμ. was not compatible with homogeneity. Their material, though rich in retinene, was clearly impure.

A recent preliminary paper by van Dorp & Arens (1947) describes an alternative synthesis of vitamin A aldehyde in impure form.

Orientating experiments in which the present authors were assisted by S. Roberts showed that potassium permanganate was the most suitable oxidizing agent and that variables such as temperature, concentration of reactants, relative volumes of phases, emulsification, etc. were of importance. Some retinene was always obtained, but yields were generally poor and not very reproducible. The one consistent tendency seemed to be an improved yield when hydrated manganese dioxide was formed, i.e. when the sulphuric acid used was insufficient to give a clear solution. An apparatus was then made in which small droplets of a solution of vitamin A in light petroleum were allowed to ascend a tall column of permanganate solution. Reaction between vitamin A and permanganate was very sluggish and it seemed possible that retinene could not be formed until some manganese dioxide was present.

Some years before Wald discovered retinene, one of us in collaboration with Sir Jack Drummond had studied the fate of vitamin A concentrates left to stand over various solids. Nothing came of the work at the time, but re-examination of the working notebooks showed that a colour-test maximum had been seen at 664 mμ. in a solution which had been in contact with manganese dioxide. A feeble band in the same position had been seen in tests with residues from the purification of carotene. At that time the mechanism of the antimony trichloride colour test was even more uncertain than it is now, and a maximum at 664 mμ. conveyed no more than a hint of some unknown impurity or artefact. When, however, the 664 mμ. band became a 'label' for retinene, the old observations slipped into a pattern. It was therefore decided to investigate the adsorption of vitamin A from inert solvents on solid oxidizing agents, in the hope of limiting the attack to the primary-alcohol grouping.

The outcome has been a smooth conversion to retinene at room temperature when vitamin A in light petroleum was allowed to stand over manganese dioxide.

EXPERIMENTAL

Materials and apparatus

Vitamin A. Two types of preparation were used:

(1) A vitamin A alcohol concentrate, from the British Drug Houses Ltd., of $E^{1\%}_{1\,\text{cm.}}$ 325 mμ. 600 (*c.* 1,000,000 i.u./g.).

(2) A vitamin concentrate prepared in the laboratory from a rich halibut-liver oil. The non-saponifiable matter was dissolved in methanol and most of the sterols were crystallized out at −35°. The filtrate was freed from solvent and the residue showed $E^{1\%}_{1\,\text{cm.}}$ 326 mμ. 900, indicating the presence of more than 50% vitamin A alcohol.

Manganese dioxide. Three types of material were used:

Sample A was the ordinary granular laboratory reagent.

Sample B was a much finer grade of the commercial product, but differed from *A* only in the size of the granules.

Sample C was prepared by mixing aqueous solutions of equivalent amounts of $MnSO_4$ and $KMnO_4$, filtering, washing until free from sulphate ions and drying on a porous plate in a desiccator.

Light petroleum. The ordinary material, b.p. 40–60°, was mainly used, but for some of the work a 60–80° fraction purified for spectroscopy was substituted.

Alumina. Small quantities of 'Brockman' activated alumina (Merck) were at first available. A material purchased from Savory and Moore Ltd. proved satisfactory, but the greater part of the work was carried out using Grade 0 activated alumina (Peter Spence and Co.) weakened by incorporation of water to the extent of 10% (w/w) just before preparing the column.

Antimony trichloride reagent. For most of the work a saturated solution of anhydrous antimony trichloride in B.P. chloroform was used, the solvent having been previously left to stand over $CaCl_2$ to remove ethanol.

Ultraviolet-absorption spectra were determined by means of the Beckman quartz photoelectric spectrophotometer and the colour-test observations were made visually using a Hilger-Nutting spectrophotometer.

Preparation of retinene

A convenient amount (0·2 g.) of vitamin A concentrate ($E^{1\%}_{1\,\text{cm.}}$ 325 mμ. 600) is dissolved in light petroleum (100 ml.) in a 250 ml. conical flask, and a calculated quantity of manganese dioxide (5 g. sample *A*) added. The weight of oxide to be used depends upon its state of subdivision, and is arrived at by a few small-scale preliminary experiments. The flask is tightly corked to prevent escape of solvent and is left in the dark at room temperature. The mixture is shaken occasionally (but this is not essential) and at the end of 6–10 days the transformation to retinene is almost complete. Longer periods of standing reduce the yield of retinene. The solution is now filtered and the manganese dioxide is washed once with light petroleum. Yields up to 80% calculated on the vitamin A used have been obtained. For example, in one experiment the product exhibited an almost symmetrical absorption curve with a maximum at 368 mμ. ($E^{1\%}_{1\,\text{cm.}}$ 390). With the antimony trichloride reagent a blue solution was obtained, $\lambda_{\text{max.}}$ 664 mμ. By applying the correction procedure of Morton & Stubbs (1946) the contribution attributable to unchanged vitamin A was $E^{1\%}_{1\,\text{cm.}}$ 325 mμ. 50, and that due to retinene was $E^{1\%}_{1\,\text{cm.}}$ 367 mμ. 382. The retinene is purified by chromatographic adsorption on slightly weakened alumina (*v.s.*), and fractions exhibiting $E^{1\%}_{1\,\text{cm.}}$ 365 mμ. 1250 (in light petroleum) are regularly obtainable.

As the maximum extinction coefficients of vitamin A and retinene do not differ greatly in light petroleum, the results imply 75% conversion and contamination of the product by about 10% of unchanged vitamin A.

The effect of agitation. A 0·5 g. portion of a concentrate, containing about 0·16 g. vitamin A alcohol, was dissolved in 250 ml. light petroleum; 100 ml. of the solution and 5 g. MnO_2 (sample *A*) were placed in a glass-stoppered bottle of 250 ml. capacity. The mixture was subjected to brisk but not violent agitation on a mechanical shaker for 8 hr. daily. A similar mixture was left to stand in the dark without shaking. After 6 days, measured portions of the two solutions were removed, and the blue solutions obtained with the antimony trichloride reagent were studied quantitatively using the wavelengths 617 and 664 mμ. characteristic respectively of vitamin A and retinene and afforded the following results:

	$E^{1\%}_{1\,\text{cm.}}$ at 617 mμ.	$E^{1\%}_{1\,\text{cm.}}$ at 664 mμ.
Without agitation	754	554
With agitation	768	710

Agitation thus resulted in an appreciably improved yield of retinene. The apparent increase in vitamin A in the second case was due to the fact that, although the retinene colour-test absorption band shows a sharp maximum near 664 mμ., there is also considerable absorption at 617 mμ. This becomes superimposed upon that from residual vitamin A, so that the intensity of absorption at 617 mμ. leads to an overestimate of the vitamin A present.

The effect of agitation is reproducible, but one further experiment requires to be mentioned. In this, a Waring Blendor was used to secure extremely vigorous agitation. Under normal conditions several hours are required for the appearance of the retinene spectrum, but in this case considerable amounts of retinene appeared within 1·5 hr. The heat generated by the very rapid stirring caused much evaporation of the light petroleum. The use of the Blendor was discontinued as being unnecessarily hazardous. For work on a larger scale, vigorous stirring in a suitably designed apparatus may well be worth while.

Rate of production of retinene. Many experiments on the time factor were carried out. The following is typical: 0·2 g. of concentrate (0·06 g. vitamin A) was dissolved in 100 ml. of purified light petroleum and the solution was left over manganese dioxide (5 g. sample *A*) in the dark. Sample portions were withdrawn at intervals, appropriately diluted, and examined spectrophotometrically. The curves shown in Figs. 1–4 exhibit a steady decrease in the intensity of the vitamin A maximum with a corresponding increase in absorption at 369 mμ.

The reaction can also be followed spectrographically. Figs. 1–4 indicate how the absorption spectrum of a test solution gradually widens and moves towards longer wave lengths as retinene becomes the predominating solute. Applying the correction procedure (Morton & Stubbs, 1946) to these curves, the change in vitamin A concentration in the solution can be determined. Using data obtained for crystalline retinene a similar correction can be used to compute the corresponding retinene concentration. The sum of the two computed curves is not identical with the observed curve. The difference curve is wide and of indeterminate structure; its origin is unknown.

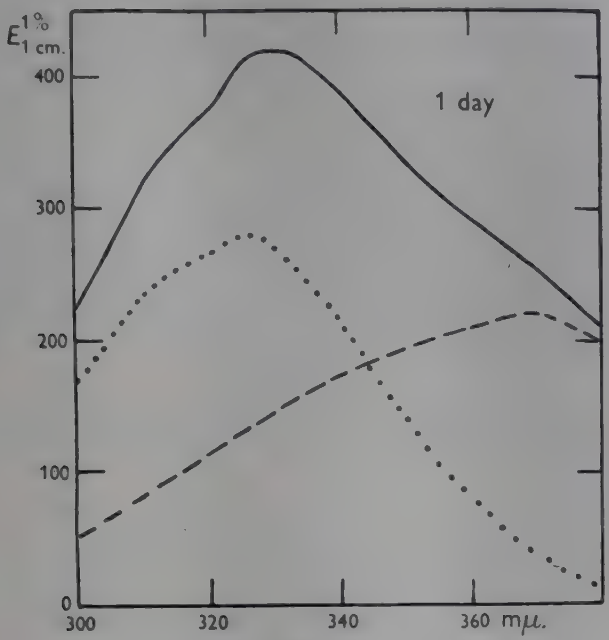


Fig. 1.

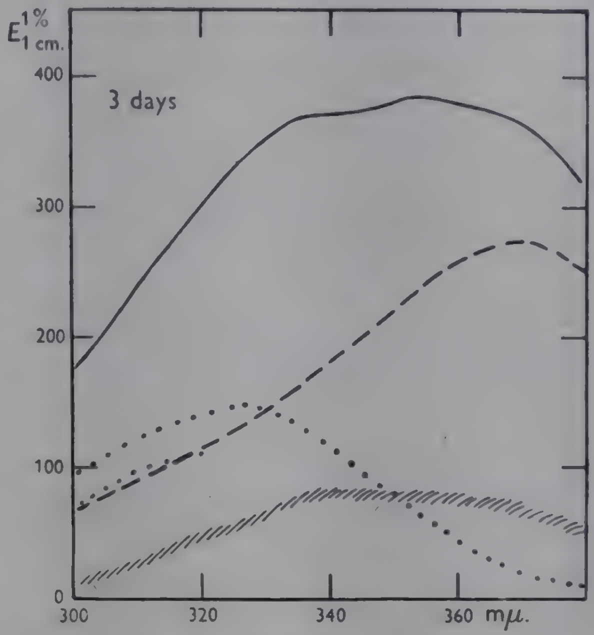


Fig. 2.

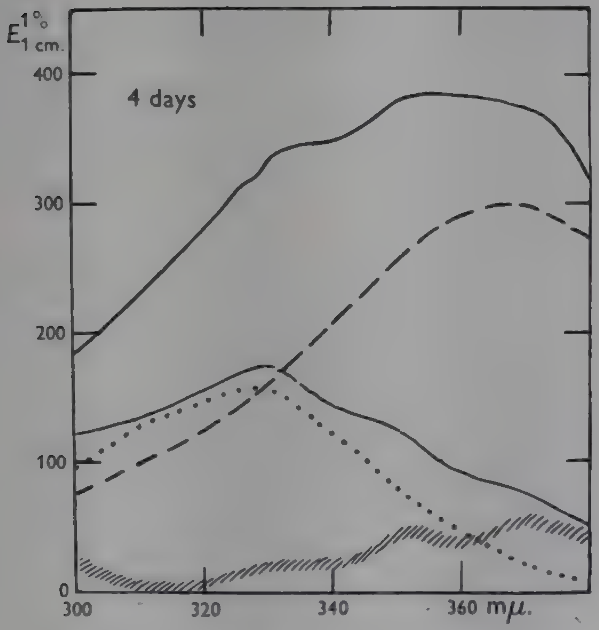


Fig. 3.

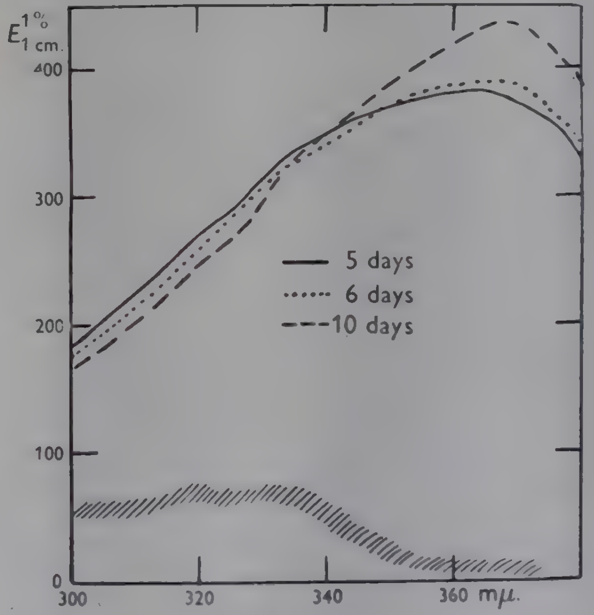


Fig. 4.

Figs. 1–4. Absorption curves obtained when a vitamin A concentrate is left over manganese dioxide in the dark (solvent, light petroleum) for periods of 1–10 days. The vitamin A band is gradually replaced by the absorption due to retinene$_1$ (measurements at intervals of 5 mμ. except around maxima where intervals were 0·5 mμ.). Observed absorption ——; vitamin A by correction procedure ·····; retinene by correction procedure - - - - - -; residual absorption after correcting for vitamin A and retinene //////.

Relative amounts of vitamin A, manganese dioxide and petroleum. The amount of manganese dioxide required for the best yield is critical and depends upon two factors: (*a*) the state of subdivision of the sample, and (*b*) the absolute amount of vitamin A in the reacting solution. Tests have been made with three different samples and good results have been obtained with each. It is, however, clear that the finer the state of subdivision, the smaller is the amount of oxide needed for the maximum yield of retinene. Thus with 1 g. of concentrate (0·3 g. vitamin) in 100 ml. of light petroleum, the best results were obtained with 25 g. of sample *A*, 5 g. of sample *B* and 1 g. of sample *C* (*v.s.*). The use of too little manganese dioxide makes the reaction slower than it need be, and the final yield is then below the best. Too much oxide also adversely affects the yield. Table 2 records an experiment in which solutions of a vitamin A concentrate in light petroleum (purified for spectroscopy) were prepared and 100 ml. portions left over manganese dioxide in the dark.

Table 2. *Effect of vitamin A concentration and amount of manganese dioxide on yield of retinene*

Vitamin A concentrate in petroleum (%)	Manganese dioxide		$E^{1\%}_{1\,\text{cm.}}$ 664 mμ. after 6 days
	Wt. (g.)	Sample	
0·2	5	*A*	756
0·2	5	*B*	335
0·5	5	*A*	493
1·0	5	*A*	393
1·0	25	*A*	702

These and other experiments show that for a given sample of manganese dioxide the ratio weight of manganese dioxide/weight of vitamin A is of great importance. The actual concentration of vitamin A (i.e. the amount of solvent) used was found to be much less critical.

The effects of light and of temperature. Light exerts a deleterious effect for both vitamin A and retinene are slowly destroyed in diffuse daylight. Experiments carried out in ordinary glassware kept away from direct sunlight gave very poor yields of retinene compared with similar experiments where exposure to daylight was reduced to a minimum. Other tests in which the reaction mixture was kept at 37° in the dark gave extremely poor results compared with control experiments carried out at room temperature. Warmth and light are therefore to be avoided.

Substitution of other oxidizing agents. Replacement of light petroleum by diethyl ether. Various solid oxidizing agents were tried as substitutes for manganese dioxide, but in no case could any retinene be observed. Chromium sesquioxide, chromic oxide, barium peroxide, lead peroxide, lead tetroxide and silver oxide all proved useless, and when portions of each were mixed with manganese dioxide, the yield of retinene could be accounted for by the MnO_2 moiety.

If vitamin A in light petroleum is left for long periods (e.g. 35 days) over solid oxidizing agents, other than manganese dioxide, vitamin A tends to disappear and to be replaced by a product, not retinene, exhibiting an absorption maximum near 280 mμ.

When purified diethyl ether was tried as solvent instead of light petroleum, the sole reaction product identified (and that in poor yield) was cyclized or anhydro-vitamin A.

Specificity of the oxidation. Manganese dioxide was left in the dark with light petroleum solutions of *iso*propyl alcohol, benzyl alcohol, octadecyl alcohol, cetyl alcohol and a mixture of C_{20} alcohols kindly supplied by Prof. T. P. Hilditch. In no case did chemical or spectroscopic tests for aldehydes give positive results.

Solutions of vitamin A esters in light petroleum showed little change after standing over manganese dioxide.

Ergosterol undergoes changes not yet fully elucidated, but calciferol is not readily attacked.

Purification of retinene

When the interaction between manganese dioxide and vitamin A is almost completed the mixture is filtered and the filtrate is poured on to a column of activated alumina. The best results are obtained in our experience by the use of slightly weakened alumina prepared as previously described. The chromatogram is developed by means of light petroleum:

Fraction 1. A yellow zone passes quickly down the column. The material gives a 617 mμ. band with the $SbCl_3$ reagent. It may contain a small amount of cyclized vitamin A and any vitamin A ester present in the original concentrate.

Fraction 2 travels more slowly as a broad brown zone containing most of the retinene.

Fraction 3 is a more strongly held pale-yellow zone. With the $SbCl_3$ reagent the eluate from this zone shows maxima at 617 and 664 mμ.

Fraction 4 is very firmly adsorbed. It remains as a brown zone at the top of the column and can only be removed by elution with ether or chloroform. The fraction shows no characteristic ultraviolet-absorption spectrum and the dirty red solution formed with the $SbCl_3$ reagent exhibits no well-defined maximum.

Fraction 2 is now reduced to small bulk by distilling off much of the solvent. The concentrated solution is rechromatrographed on weakened alumina. The retinene fraction is collected and examined spectroscopically. The ultraviolet absorption is maximal at 369 mμ. (in petroleum) and a value of $E^{1\%}_{1\,\text{cm.}}$ 1250 is regularly obtained. This seems to be the limit of purification attainable by simple chromatography.

It is important that the alumina should not be too active. Ordinary 'Brockman' alumina is satisfactory but the solvent percolates rather slowly, whilst the grade 0 (Peter Spence and Co.) alumina as supplied tends to produce a red artefact from retinene ($\lambda_{\text{max.}}$ 290 mμ.; $SbCl_3$ test $\lambda_{\text{max.}}$ 572→610 mμ.). By using weakened alumina the chromatography is speeded up and decomposition of retinene is eliminated.

Examination of used manganese dioxide. After filtering the reaction mixture, the manganese dioxide retains some adsorbed material which can be eluted with chloroform. The solution gives a blue colour with the Carr-Price reagent with maxima at 573, 610 and 664 mμ. If the chloroform is removed and replaced by light petroleum, ultraviolet maxima at 290 and 365 mμ. are recorded. It seems that some retinene and some of the red artefact are adsorbed on the manganese dioxide.

Crystallization of retinene

Purified light petroleum was found to be the best solvent for crystallization. The solute was a retinene fraction $E^{1\%}_{1\,\text{cm.}}$ 369 mμ. 1250 and a concentration of about 5% (w/v) was used for a preliminary cooling to −72° for 48 hr., when a small yield of feathery white crystals was obtained. This material (which was discarded) showed an absorption band at 270 mμ. in light petroleum or in chloroform, and with the $SbCl_3$ reagent a rose-coloured solution was obtained showing continuous end absorption over the whole visible spectrum range.

In addition to the above material very small amounts of a substance (also discarded) with a band of comparatively low intensity at 348 mμ. in petroleum or in chloroform were obtained. The $SbCl_3$ reagent gave a blue-coloured solution with absorption bands of approximately equal intensity at 664 and 617 mμ.

After removal of these impurities by filtering through a Buchner funnel cooled with solid carbon dioxide, the mother liquor was concentrated *in vacuo* to one-fourth its original bulk. The concentrated solution was again cooled to −72° for 24 hr. when a brown semi-solid mass was obtained. The mother liquor was decanted off and the residue dissolved in freshly distilled light petroleum to give a 5% (w/v) solution. This was kept at −72° for 10 days when a large crop of reddish brown crystals, m.p. 56·5–58°, was obtained which showed an absorption band with a single maximum at 373 mμ. in *cyclo*hexane, $E^{1\%}_{1\,\text{cm.}}$ 1354.

The material so obtained was recrystallized very carefully from 5% (w/v) solution in light petroleum, the solution being cooled slowly from 0° to −40° over a period of 7 hr. The mixture was then left at −72° for 24 hr. when large orange-red crystals were obtained in good yield. The overall yield (of crystallization) was about 20%. The crystals are large clusters predominantly needle-like in shape and melt at 61–62°. (Found: C, 84·2; H, 9·76%; mol. wt. (Rast) 236. $C_{20}H_{28}O$ requires C, 84·5; H, 9·85%; mol. wt. 284.)

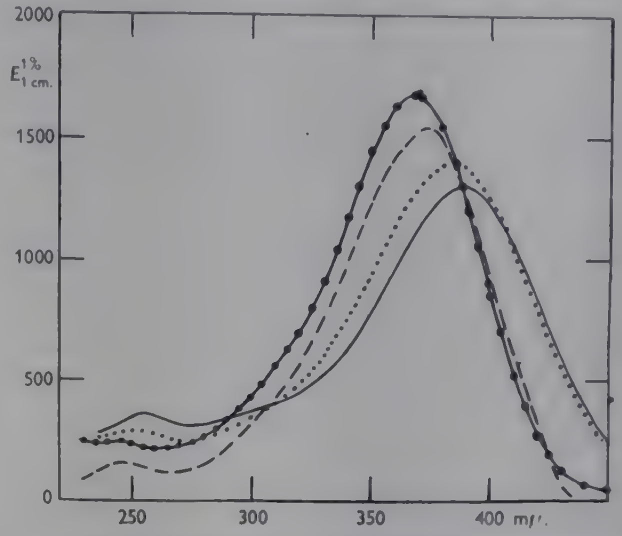


Fig. 5. Absorption spectrum of retinene$_1$ in light petroleum ●—●; *cyclo*hexane — — —; ethanol ······; and chloroform ——.

Table 3. *Absorption maxima of retinene in various solvents*

Solvent	$\lambda_{\text{max.}}$ (mμ.)	$E^{1\%}_{1\,\text{cm.}}$
Light petroleum (40–60°)	369·5	1685
*cyclo*Hexane	373	1548
Ethanol	385·5	1400
Chloroform	389	1303

The absorption spectra of crystalline retinene have been determined in various solvents. The full curves are recorded in Fig. 5 and the maximum intensity of absorption is recorded in Table 3, e.g. in *cyclo*hexane,

$$E^{1\%}_{1\,\text{cm.}}\ 373\ \text{m}\mu.,\ 1548;\quad E^{1\%}_{1\,\text{cm.}}\ 245\ \text{m}\mu.,\ 159.$$

With the antimony trichloride reagent a blue-coloured solution was obtained, giving a single absorption band at 664 mμ., with an $E^{1\%}_{1\,\text{cm.}}$ value of 3400.

Derivatives of retinene

Retinene-2:4-dinitrophenylhydrazone. An ethanolic solution of 2:4-dinitrophenylhydrazine sulphate was added to an equivalent amount of retinene in ethanol, when an immediate darkening of the solution was observed. From this solution a good crop of crystals was obtained and the material was crystallized from ethanol and from acetone. Reddish brown needles were obtained with m.p. 207–208°. (Found: C, 66·8; H, 6·9; N, 12·2%; mol. wt. (Rast) 336. Calc. for $C_{26}H_{32}N_4O_4$: C, 67·2; H, 6·9; N, 12·1%; mol. wt. 464.) The spectrum of the derivative showed bands at 442 and 260 mμ. with $E^{1\%}_{1\,\text{cm.}}$ 1166 and 346 respectively.

Retinene semicarbazone. This derivative was prepared by adding an aqueous ethanolic solution of semicarbazide hydrochloride to an ethanolic solution of retinene. The mixture was warmed for a few minutes, and after standing overnight was extracted with ether and the semicarbazone crystallized from a mixture of ether and light petroleum. Although work has not yet been completed on this material, one sample of the derivative, which must have been of high purity, had m.p. 161–164° and an absorption band at 385 mμ. in chloroform with $E^{1\%}_{1\,\text{cm.}}$ 1742. On standing in solution at 0° for some weeks, the intensity of the 385 mμ. band had decreased with the production of another band of equal intensity at 365 mμ. These data are at variance with those reported by van Dorp & Arens (1947) who state that this derivative has m.p. 207–209° (with decomposition), an absorption spectrum $\lambda_{\text{max.}}$ 375 mμ., log $E_{\text{max.}}$ 4·87, and reacts in dilute solutions with chloroformic $SbCl_3$ to give a purple-red colour and in stronger solutions a blue-violet colour. Comparison with our data is difficult for they do not state the solvent used to determine the ultraviolet spectrum and the colour-test statements are too qualitative.

Retinene hydrazone derivatives. Two hydrazone derivatives of retinene have been obtained by reaction of a solution of hydrazine hydrate with retinene followed by fractional crystallization. Work has not yet been completed but the following details have been obtained.

Derivative A, m.p. 108–109°. Single broad band absorption maximum in ethanolic solution.

$\lambda_{\text{max.}}$ 355 mμ.; $E^{1\%}_{1\,\text{cm.}}$ 280.

Derivative B, m.p. 135–145°. Two absorption bands in ethanolic solution.

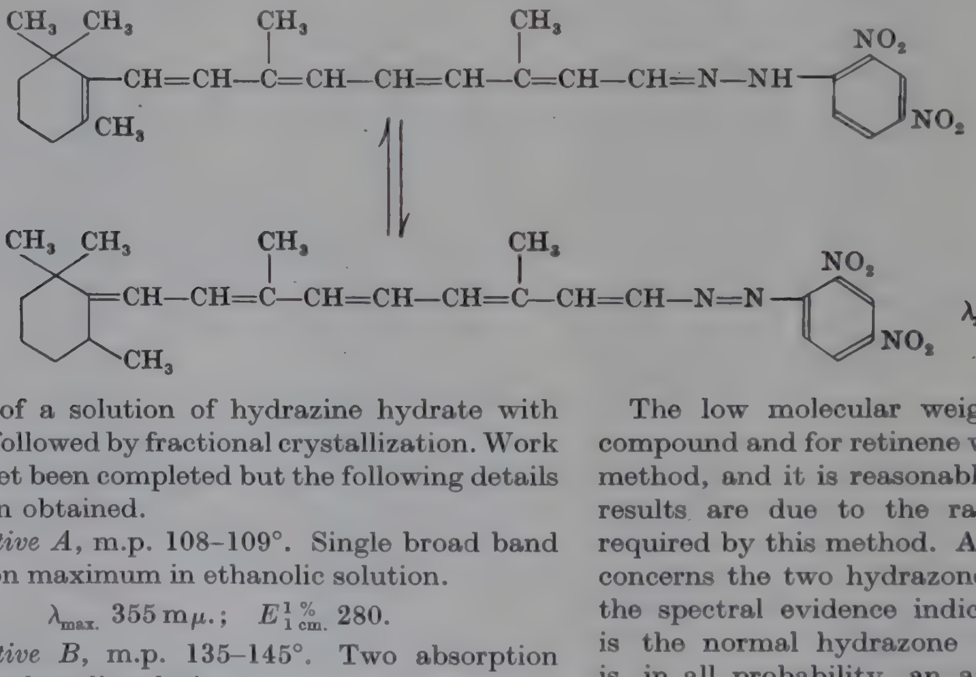
$\lambda_{\text{max.}}$ 460 mμ.; $E^{1\%}_{1\,\text{cm.}}$ 1502
298 mμ.; 322.

With pure retinene, a semicarbazone, m.p. 193–195° and $E^{1\%}_{1\,\text{cm.}}$ 385 mμ. 2062, and a 'hydrazone', m.p. 177°, $\lambda\lambda_{\text{max.}}$ 462 and 297 mμ., $E^{1\%}_{1\,\text{cm.}}$ 1930 and 334 respectively, have since been obtained. The semicarbazone analyzed correctly. Found: C, 73·8; H, 9·1; N, 12·3. Calc. for $C_{21}H_{31}N_3O$: C, 73·0; H, 9·1; N, 12·5%. The '*hydrazone*' analyzed correctly for an azine. Found: C, 85·1; H, 10·0; N, 4·74; mol. wt. 500. $C_{40}H_{56}N_2$ requires C, 85·1; H, 9·9; N, 4·9%; mol. wt. 564.

DISCUSSION

The preparation described above forms a convenient and economical method of preparing retinene. The material so formed shows the same spectral characteristics as that isolated from retinas by Wald (1935–6), and differs from the materials prepared by Morton & Goodwin (1944) and by Hunter & Hawkins (1944) only in its state of purity. Hunter & Hawkins obtained a material showing absorption bands at 368 and 350 mμ. but it is now apparent that the 350 mμ. band is not characteristic of retinene but is due to an impurity, probably an artefact. This artefact has been isolated in an impure state and in small yield and may possibly be a polymerization product of retinene.

The preparation of the retinene crystals and of its derivatives gives proof to the suggestion of Morton (1944) that retinene is the aldehyde of vitamin A. Some points of doubt, however, still remain to be investigated completely, e.g. the absorption spectrum of retinene-2:4-dinitrophenylhydrazone is atypical, though it is possible that the following equilibrium may exist:


CH₃ CH₃
CH₃
CH₃
—CH=CH—C=CH—CH=CH—C=CH—CH=N—NH—
NO₂
NO₂
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CH₃ CH₃
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CH₃
=CH—CH=C—CH=CH—CH=C—CH=CH—N=N—
NO₂
NO₂
CH₃

$\lambda_{\text{max.}}$ c. 360 mμ.

$\lambda_{\text{max.}}$ c. 440 mμ.

The low molecular weights obtained for this compound and for retinene were obtained by Rast's method, and it is reasonable to believe that these results are due to the rather severe treatment required by this method. A further point of doubt concerns the two hydrazone derivatives, although the spectral evidence indicates that *derivative A* is the normal hydrazone type and *derivative B* is, in all probability, an azine compound of the type

$$R\text{—CH}=\text{CH—CH}=\text{N—N}=\text{CH—CH}=\text{CH—}R.$$

The semicarbazone derivative prepared seems normal, and the production of the 365 mμ. band on standing in solution can be explained by assuming the existence of the following dynamic equilibrium (see p. 523).

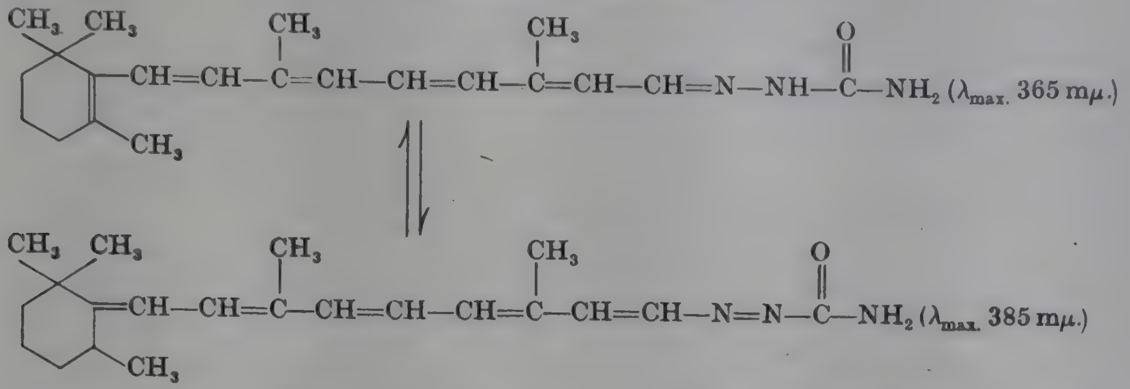
The conversion of vitamin A alcohol to retinene depends upon the surface catalysis of manganese dioxide. In all probability the process is triphasic, the three stages comprising:

(1) The removal of vitamin A alcohol from the light petroleum phase by adsorption on manganese dioxide.

(2) The conversion of the CH_2OH group to the CHO group.

(3) The passage of retinene into the petroleum phase.

The oxidation, as far as our investigations go, is specific, for replacement of either of the reactants (i.e. vitamin A or manganese dioxide) by a similar substance prevents any comparable reaction. The oxidation process is dependent on two facts, viz. that vitamin A is more strongly adsorbed on manganese dioxide than is retinene, and that the solid adsorbent is capable of converting the primary-alcoholic group to the aldehyde group without attacking other parts of the molecule. Apart from the importance of the reaction from a purely preparative point of view, it may well be that it is of deeper significance. The mechanism connecting vitamin A with retinene in the cycle of scotopic vision is a matter of some doubt, but it is possible that one stage of the retinal process is a surface oxidation analogous to the one described above.


CH₃ CH₃
CH₃
CH₃
O
—CH=CH—C=CH—CH=CH—C=CH—CH=N—NH—C—NH₂ (λmax. 365 mμ.)
CH₃
CH₃ CH₃
CH₃
CH₃
O
=CH—CH=C—CH=CH—CH=C—CH=CH—N=N—C—NH₂ (λmax. 385 mμ.)
CH₃

SUMMARY

1. A simple method of preparing retinene (vitamin A aldehyde) from vitamin A in excellent yield is described.

2. Retinene has been obtained crystalline and fully characterized.

3. The preparation of derivatives of retinene with 2:4-dinitrophenylhydrazine, hydrazine and semicarbazide is described.

4. Absorption spectra of retinene and its derivatives are given.

We are indebted to the Medical Research Council and the Ministry of Food for financial assistance.

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Proteolytic Activity of Preparations of Crystallized Ribonuclease

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The first preparations of ribonuclease were made by methods involving heating (Jones, 1920; Dubos & Thompson, 1938), but since the adoption of the Kunitz (1940) method for the isolation of the enzyme in crystalline form, this step has been omitted. It appears to have been assumed that crystallinity presupposes homogeneity, and crystallized preparations of the enzyme, which have never been exposed to heat, have been used by many workers under conditions in which the presence of other enzymes, and of protease in particular, renders interpretation of results uncertain. It has been established with many proteins that crystallinity is no guarantee of purity (Pirie, 1940) and this seems to apply to ribonuclease. Cohen (1945), when studying the effect of proteolytic enzymes on a thymus nucleohistone, and Stowell & Zorzoli (1947), using ribonuclease in cytochemical work, detected proteolytic activity in the ribonuclease crystallized by Kunitz's method. Cohen stated that it is impossible to decide whether ribonuclease itself possesses proteolytic activity or whether the crystalline preparations were contaminated with protease.

In attempts to determine the action of ribonuclease on plant viruses, many different crystalline preparations of the enzyme have been prepared. All these have been found to possess proteolytic activity, but the ratio of ribonuclease to proteolytic activity has varied in different preparations, although all of them have otherwise seemed similar. Hence it seems more likely that the preparations are mixtures of enzymes rather than that ribonuclease itself has proteolytic activity.

The simultaneous occurrence of proteolysis and ribonuclease activity could, of course, invalidate many of the results obtained in cytochemical work, or with such substrates as potato virus X, which are affected by proteolytic enzymes. For the unequivocal interpretation of results with ribonuclease, it is essential that proteolytic activity should be excluded, and this paper describes methods whereby this can be achieved. It also describes the effect of pepsin and chymotrypsin on ribonuclease, as well as the effect of the protease present in its crystallized preparations.

MATERIAL AND METHODS

Enzymes. Ribonuclease was prepared from beef pancreas and crystallized from $(NH_4)_2SO_4$ solutions by the method of Kunitz (1940). Small amounts of the enzyme could also be obtained in crystalline form by complete saturation with $(NH_4)_2SO_4$ of the filtrates which are obtained after precipitation of most of the enzyme at 80% saturation. The enzyme was usually crystallized twice, dissolved in, and dialyzed against, distilled water, and then dried in flat dishes in a desiccator at 37°. It was stored as a dry powder, which dissolved readily in water or in buffer solutions.

Crystalline chymotrypsin was prepared from beef pancreas by the method described by Northrop (1939). It was crystallized eight times as chymotrypsinogen, then converted into chymotrypsin and twice crystallized. The crystals were dissolved and dialyzed against distilled water, dried and stored as a powder.

The pepsin used was a commercial preparation obtained from the British Drug Houses Ltd.

The following buffer solutions were used: pH 2·0–4·9, 0·1 M-Sorensen citrate-HCl; pH 4·9–6·6, 0·1 M-Sorensen citrate-NaOH; pH 6·8–8·0, 0·2 M-boric acid-borax.

Determination of enzyme activities. Ribonuclease activity was measured by estimating the amounts of yeast nucleic acid rendered non-precipitable by acetic acid according to the method previously described (Kleczkowski, 1946). A commercial preparation of yeast nucleic acid (British Drug Houses Ltd.) was used for the purpose; about 40% of the nucleic acid was precipitated when 10 vol. of glacial acetic acid were added to 1 vol. of a neutral solution.

Proteolytic activity was measured by estimating the amount of casein rendered soluble in 1% trichloroacetic acid. One vol. of the solution under test was mixed with 2 vol. of a 1% solution of casein and the mixtures incubated at 37°. Trichloroacetic acid (10% w/v) was then added to bring the concentration to 1%. The precipitates were centrifuged down and the nitrogen in the supernatant fluids was estimated. If the mixtures were incubated at pH values other than 6–7, they were neutralized before adding trichloroacetic acid. Enzyme concentrations and times of incubation varied in different experiments.

Other determinations. Phosphorus and nitrogen determinations were made by the methods previously described (Kleczkowski, 1946).

EXPERIMENTAL

The effect of pH *on the ribonuclease and proteolytic activities of preparations of ribonuclease*

The identity of the protease contaminating crystalline preparations of ribonuclease is uncertain, but its pH range of activity suggests similarities with trypsin and chymotrypsin. When tested at pH 7·2 with casein as substrate, 1 mg. of an average preparation of ribonuclease usually has a proteolytic effect equivalent to that of 0·02 mg. of crystalline chymotrypsin. Thus if the contaminating protease were chymotrypsin, it would be present in ribonuclease to the extent of about 2%, and less if it were trypsin.

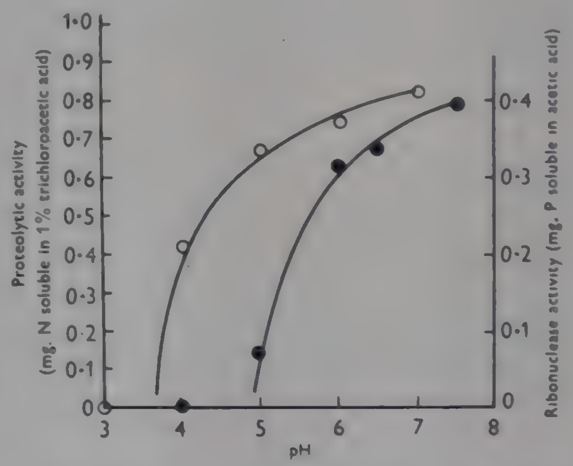

Fig. 1. The effect of pH on proteolytic and ribonuclease activities of a ribonuclease preparation. Proteolytic activity: 1 ml. of 0·1% ribonuclease in water was mixed with 2 ml. of 1% casein solution in a buffer of required pH, and the mixtures were incubated for 24 hr. at 37°. Ribonuclease activity: 0·5 ml. of 0·001% ribonuclease solution in a buffer of required pH was mixed with 0·5 ml. of 3% solution of yeast nucleic acid in the same buffer, and the mixtures were incubated for 30 min. at 50°. ○—○, ribonuclease activity; ●—●, proteolytic activity.

The effect of pH on the proteolytic and ribonuclease activities of a representative ribonuclease preparation is shown in Fig. 1. No ribonuclease activity could be detected at pH 3, but it became appreciable between pH 3 and 4, and increased rapidly with increasing pH values. There was no detectable proteolytic activity at pH 4; it appeared as the pH approached 5, and increased rapidly above pH 5. It is thus possible to obtain a relatively slight ribonuclease activity in the absence of proteolytic activity by incubating in the pH range 4·0–4·5.

The effect of heat on the proteolytic and ribonuclease activities of preparations of ribonuclease

The earlier work on the preparation of ribonuclease paid little attention to the conditions of heating. Jones (1920) boiled aqueous extracts of pig's pancreas, and Dubos & Thompson (1938), after extracting commercial pancreatin with 50% acetone, precipitated the enzyme by adding more acetone, dissolved the precipitated enzyme in water and heated it in boiling water. No control of the pH seems to have been made during heating, and no tests are described for the effect of heating on proteolytic activity. After Kunitz (1940) had crystallized the enzyme, he estimated its heat stability over a range of pH values, but did not test his preparations for enzymic activities other than ribonuclease activity.

The effects of heat on the proteolytic and ribonuclease activities were investigated as follows. Tubes of 15 mm. diameter, containing 2 ml. of 0·1% ribonuclease in buffers at different pH values, were immersed in boiling water for 5 min. To prevent evaporation, the tubes were closed with rubber stoppers through which capillary tubes were inserted. The temperature of the heated fluids did not reach 100°, but increased in the following way after immersion in the water bath: after 1 min., 90°; 2 min., 96·5°; 3 min., 98·5°; 4 min., 99·0°; 5 min., 99·25°. After 5 min., the tubes were rapidly cooled, the pH in all was adjusted to 7 and the volume to 4 ml.; the proteolytic and ribonuclease activities of the solutions were then determined.

It will be seen from Fig. 2 that at pH values between 2 and 3 proteolytic activity was less affected by heat than was ribonuclease activity; as the pH increased the susceptibility of proteolytic activity to heat increased progressively, whereas that of ribonuclease remained apparently unchanged until the pH reached about 6, after which it increased rapidly. After heating between pH 7·0 and 8·0 proteolytic activity could not be detected, whereas ribonuclease activity, though decreased, was still present; this also disappeared as a result of heating at pH 8·0.

Somewhat different results may be obtained by changing the conditions of heating. For example, by varying the volumes of heated ribonuclease solutions or the diameter of tubes in which they are heated, the rate of temperature increase is changed, and as a result the drop in ribonuclease activity is different. The difference is particularly pronounced in the pH range where the rate of inactivation of the enzyme is relatively high, i.e. at pH values higher than 6. The dependence of heat inactivation of ribonuclease on the pH, as shown in Fig. 2, is on the whole in agreement with the results obtained by Kunitz (1940). The differences, notably the much greater inactivation at pH values higher than 6 found by Kunitz, are almost certainly due to different conditions of heating.

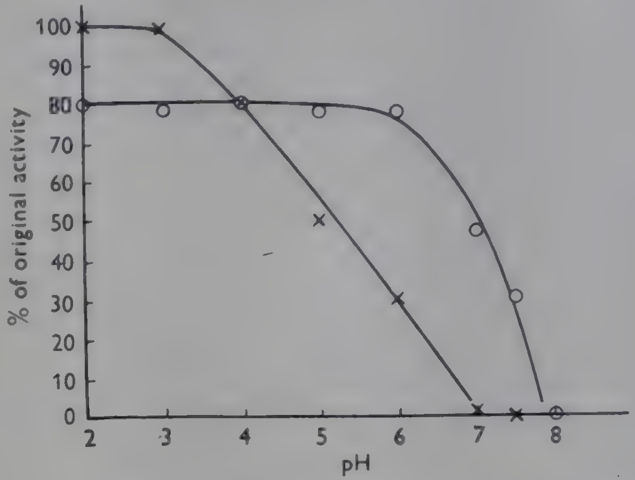

Fig. 2. The effect of pH on heat stability of proteolytic and ribonuclease activities of a preparation of ribonuclease. ○—○, ribonuclease activity; ×—×, proteolytic activity.

These results suggested that suitable conditions of heating might be found that would destroy proteolytic activity while leaving satisfactorily active preparations of ribonuclease. The effect of heating was therefore studied in greater detail.

When tubes of 15 mm. diameter, containing 2 ml. of 0·1% ribonuclease solution in 0·2M-boric acid-borax buffer at pH 7·5, were immersed for 10 min. in a water bath at 80°, ribonuclease activity was reduced by only 20%, whereas the proteolytic activity was so reduced that it was not possible to estimate it accurately by the method described above. When 1 ml. of a 0·1% solution of heated ribonuclease was incubated with 2 ml. of 1% casein solution at pH 7·5 for 24 hr. at 37° it failed to render any measurable amount of the casein non-precipitable by 1% trichloroacetic acid. Some residual proteolytic activity remained, however, and it could be measured by using the more accurate method of Pope & Stevens (1939) as modified by Tracey (1948). This method is based on the decomposition of a suspension of copper phosphate by the amino groups liberated from the protein substrate (gelatin). The estimations were kindly made by Mr M. V. Tracey.

After heating in a water bath at 80° under the conditions given above, the proteolytic activity of ribonuclease solutions was reduced to about one-fiftieth of that of the unheated preparation. After heating for 5 min. in boiling water in otherwise similar conditions no proteolytic activity could be detected even by the more sensitive test, whereas about 30 % of the original ribonuclease activity still remained. The heating should not be prolonged beyond 5 min., as ribonuclease activity is then further reduced; after 10 min. only 5 % of the original ribonuclease activity remains, and after 15 min. none can be detected.

No significant differences were found between the heat stabilities of crystalline and uncrystallized preparations of the enzyme, although the latter contained about ten times more proteolytic activity per unit weight. Preparations made simply by precipitation with 80 % saturated ammonium sulphate, instead of by crystallization, when heated at pH 7·5 for 5 min. in boiling water, lost all detectable proteolytic activity but retained about 30 % of their original ribonuclease activity. During heating, considerable amounts of coagulum usually separate from these preparations. Crystalline preparations, on the other hand, usually remain almost clear, though sometimes an opalescence, which can be removed by centrifugation, may develop.

In the light of these results it seems that if the heat method is applied to destroy proteolytic activity, crystallization of the enzyme is of no advantage for many kinds of purely qualitative work, particularly as the yields of amorphous enzyme by precipitation with ammonium sulphate (80 % saturated) are higher than those obtained by crystallization.

Action of unheated and heated ribonuclease on yeast nucleic acid

Solutions of unheated and of heated ribonuclease in concentrations giving equivalent activity as indicated by their ability to render yeast nucleic acid non-precipitable by acetic acid, were also compared for their ability to produce dialyzable products from yeast nucleic acid.

Three 5 ml. samples of a 3 % aqueous solution of commercial yeast nucleic acid at pH 6·0 were mixed respectively with 5 ml. of 0·001 % solution of unheated ribonuclease, with 5 ml. of 0·0033 % solution of heated ribonuclease, both in 0·2 M-boric acid-borax buffer at pH 6·8, and with 5 ml. of the buffer. (The ribonuclease was heated as a 0·1 % solution in 0·2 M-boric acid-borax buffer at pH 7·5, and was diluted 1 : 30 in the pH 6·8 buffer.) After the mixtures had been incubated for 30 min. at 50°, 1 ml. samples were taken from each and mixed with 10 ml. of glacial acetic acid. The precipitates were spun down, washed in glacial acetic acid and their P contents estimated. These were 0·200, 0·195 and 0·800 mg., respectively, in the precipitates from the mixtures with unheated ribonuclease, heated ribonuclease and with the buffer alone.

The remainder of the digests was divided into two portions of 4·5 ml. each. These were placed in separate test tubes, the openings of which were then closed with cellophan membranes held in position with rubber bands. Each tube was inverted and the membrane immersed in a wider tube containing 5 ml. water. The tubes were left for 6 hr. at room temperature and the P contents of the outside and inside fluids were estimated.

Table 1. *Formation of dialyzable products from yeast nucleic acid as a result of incubation with unheated and with heated solutions of ribonuclease*

Addition to yeast nucleic acid	Test no.	Phosphorus content (mg. P) Outside fluid	Phosphorus content (mg. P) Inside fluid
Unheated ribonuclease	1	1·23	3·75
	2	1·20	3·77
Heated ribonuclease	3	1·25	3·74
	4	1·22	3·81
Buffer (control)	5	0·44	4·51
	6	0·42	4·55

From the results shown in Table 1 it will be seen that some of the yeast nucleic acid incubated with the buffer alone was dialyzable, but the amount of dialyzable material was increased to approximately the same extent by incubation both with the unheated and heated ribonuclease. Kunitz (1940) found that his crystalline preparations of ribonuclease caused the formation of dialyzable products from yeast nucleic acid, but in an earlier work Schmidt & Levene (1938), using ribonuclease prepared from commercial pancreatin by the method of Dubos & Thompson (1938), could not detect their formation. Tipson (1945) suggested that this discrepancy might be due to the fact that Schmidt & Levene (1938) used heated enzyme preparations, whereas Kunitz used unheated enzyme. This possibility would seem to be excluded by the results described above. If no such products were formed by the preparations used by Schmidt & Levene, it seems probable that their ribonuclease was not identical with that which is obtained from fresh beef pancreas by the method of Kunitz. This cannot be assumed with certainty, however, for an alternative explanation, suggested by Loring & Carpenter (1943), is that Schmidt & Levene (1938) failed to detect any dialyzable products because these were retained by the proteins present in their enzyme preparations.

The effect of proteolytic enzymes on ribonuclease

For these experiments, the ribonuclease solutions were dissolved either in 0·1 M-citrate-HCl buffer at pH 2·5 or in 0·2 M-boric acid-borax buffer at pH 7·5, and the solutions of pepsin and of crystalline chymotrypsin, neither of which showed any detectable ribonuclease activity, were dissolved in water. The mixtures were incubated at 37°. Samples were taken at stated intervals, diluted 1 : 25 and 1 : 50 in 0·2 M-boric acid-borax buffer at pH 6·8, mixed with equal volumes of 3 % solution of yeast nucleic acid in water at pH 6·0, and incubated for 30 min. at 50°. The amounts of P in the material precipitable with acetic acid were estimated, and percentages of the ribonuclease activities were determined by comparison with control enzyme solutions, as previously described (Kleczkowski, 1946).

Table 2 summarizes the results of experiments in which the susceptibility of heated and unheated ribonuclease to proteolytic enzymes was thus tested. Both pepsin and chymotrypsin destroyed ribonuclease, the former being the more effective. No ribonuclease activity could be detected after 24 hr. incubation with pepsin, whereas one-third of the ribonuclease activity was still present after 48 hr. incubation with chymotrypsin.

Table 2. *The effect of pepsin, chymotrypsin and of ribonuclease-protease on ribonuclease*

	Contents of mixtures (ml.)						Ribonuclease activity (as % of the original) after:		
Test no.	Unheated ribonuclease (0·05 %)	Heated ribonuclease (0·1 %)	Pepsin (0·05 %)	Chymotrypsin (0·05 %)	Buffer	pH	0 hr.	24 hr.	48 hr.
1	1	—	1	—	—	2·5	100	0	—
2	1	—	—	—	1	2·5	100	100	—
3	1	—	—	1	—	7·5	100	60	30
4	1	—	—	—	1	7·5	100	85	70
5	—	1	—	1	—	7·5	100	60	30
6	—	1	—	—	1	7·5	100	100	100

It can also be seen that at pH 7·5, but not at pH 2·5, ribonuclease was hydrolyzed by the protease present in its crystalline preparation; this is shown by the fact that incubation at pH 7·5 decreased ribonuclease activity of the unheated preparation (test no. 4), but not that of the heated one (test no. 6) in which proteolytic activity had been destroyed. The ribonuclease activity of material used in tests nos. 5 and 6 corresponded to about 70 % of that used in tests nos. 1–4. When control mixtures, similar to those shown in Table 2, were stored at 2° instead of being incubated at 37°, no detectable drop in ribonuclease activity occurred in any of them.

Dubos & Thompson (1938), using preparations of ribonuclease prepared by their method from commercial pancreatin, found that ribonuclease was hydrolyzed by pepsin, but remained unaffected by trypsin or chymotrypsin. Whether their enzyme was really resistant to trypsin and chymotrypsin, or whether their failure to detect any effect was due to experimental conditions, is uncertain. If the former is correct, then it seems that their ribonuclease differs from our preparations.

SUMMARY

1. Crystalline preparations of ribonuclease invariably possessed proteolytic activity, which varied in amount in different preparations. The pH zones of proteolytic and ribonuclease activities are somewhat different, and between pH 4·0 and 4·5 only the ribonuclease activity, though far from its optimum, is demonstrable.

2. By heating at pH 7·0–7·5 under specified conditions, the proteolytic activity can be greatly reduced or destroyed, leaving between 30–80 % of the original ribonuclease activity.

3. Ribonuclease is susceptible to both pepsin and chymotrypsin, but it is hydrolyzed much more rapidly by pepsin. The proteolytic enzyme present in crystalline preparations of ribonuclease also hydrolyzes ribonuclease.

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1948

The Effects of Various Organic Materials on Soil Nitrification

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In his early work on the isolation of nitrifying organisms from various soils Winogradsky (1891) found that the organisms could not be cultured on gelatin. Winogradsky & Omeliansky (1899, 1902) showed that various organic materials (glucose, gelatin, asparagine, peptone, etc.) were actually toxic to the nitrifiers in culture. The more recent work on nitrification does not entirely confirm these findings. Kingma Boltjes (1935) observed nitrification in the presence of glucose, and succeeded in showing that the inhibitory effect of peptone was due to the presence of free amino-acids.

Hes (1937) in studying *Nitrosomonas* not only cultured his organisms on agar, but found that the addition of an egg preparation actually stimulated their growth, thus confirming the results of Fred & Davenport (1921) and of Kingma Boltjes (1935). Bömecke (1939), on the basis of the results of Warburg respiration experiments, even claimed to have shown a small but definite heterotrophic dissimilation in nitrifying cultures. Nevertheless, the vague concept that 'organic matter is toxic to nitrification', which arose from the work of Winogradsky & Omeliansky (1899), is still current in the literature on nitrification, and within the last 10 years Pandalai (1946) and Desai & Fazal-ud-Din (1937) have been led to put forward theories of symbiosis to account for the fact that nitrification proceeds in the presence of organic matter. But the situation remains obscure, and the present work was begun in order to determine whether there is in fact any need for a theory of symbiosis to account for soil nitrification and to discover whether the various organic materials reported as being toxic in culture have any direct effect on the nitrification process in soil.

METHODS

The percolation technique previously described (Lees & Quastel, 1946*a*) was used throughout, but the percolation apparatus was the simplified and improved form described by Lees (1947). Measurements of the time taken for ammonium ions, freshly added to the percolate, to reach an equilibrium with the soil exchange complexes have shown that equilibrium is reached within 3 hr. Because this equilibrium is reached so quickly, it has been possible to conduct replicable nitrification experiments of only 5 hr. duration.

The garden soil containing 0·4% organic N used in previous experiments (Lees & Quastel, 1946 *a*, *b*, *c*) has been used in all the present work. It is herein described as the allotment soil. Except where otherwise stated experiments were carried out on 10 g. of this soil (air dry and sieved to 4·0–1·0 mm. mesh) and 100 ml. percolate.

Determinations of the various forms of inorganic N were made by methods similar to those described by Lees & Quastel (1946*a*).

Of organic materials 10 ml. at 1% concentration were added to 90 ml. percolate. The gelatin and peptone solutions were dialyzed against 100 vol. distilled water for 24 hr. before use, in order to minimize the possible toxic effect of free amino-acids noted by Kingma Boltjes (1935). The 'humus' was prepared at pH 7 from a Littleport fen soil by a pyrophosphate extraction (Bremner & Lees, in course of preparation).

Calculation of results. The values of 'μg. NO_3-N formed/g. soil' given in the tables were calculated from percolate analyses by methods similar to those already described (Lees & Quastel, 1946*a*).

Because the experimental period was necessarily short, the measured formation of NO_2-N or NO_3-N in the percolates was small (only some 3–4 μg. N/ml.). The reagent blank on the phenoldisulphonic acid estimation was equivalent to about 1·0 μg. NO_3-N/ml. percolate or about 25% of the actually estimated amount of nitrate. This reagent blank seems unavoidable; it appears to be due mainly to the H_2O_2 used for oxidation of nitrite in the sample. Fortunately a reagent blank introduces only a small error into comparative colorimetric readings if all the 'test' readings are close to one another and are read against the same standard, and even this small error has been eliminated by the use of the following easily derived equation in the calculation of the results:

$$U = \frac{S\,(T-B)}{(S-B)},$$

where S = nominal concentration of standard reference solution; T = colorimetrically observed concentration of test solution X; B = colorimetrically observed concentration of blank solution; and U = true concentration of test solution X.

Experimental procedure. The design of a valid experimental procedure to test the effect of organic materials on the nitrification of ammonium ions by soil is not simple. The nitrifying organisms have a considerable lag phase in their proliferation curve; nitrification of ammonium ions percolated through the allotment soil is not appreciable during the first 3 days of the experiment and is not well established before the 6th (Lees & Quastel, 1946*a*). On the other hand heterotrophic proliferation in response to the addition of a carbon source to the percolate is likely to result in a relatively enormous immobilization of mineral N during the first 4 days (Fig. 1). This figure shows only the immobilization of NO_3-N in the presence of various organic materials, but similar curves are obtained if $(NH_4)_2SO_4$ is substituted for $NaNO_3$ in the percolate. The gradual reappearance of NO_3-N in the percolates after 4 days indicated that all the carbon of the added organic material had been consumed and that lysis of a moribund heterotrophic population was taking place. The difficulty of following nitrification (i.e. the formation of nitrite and subsequently nitrate, from ammonium ions) under these conditions was largely overcome by the use of the 'stimulated soil' technique, already described by Lees & Quastel (1946*c*), in which soils are stimulated to active nitrification before the experiment proper. Such soils begin to nitrify fresh $(NH_4)_2SO_4$ immediately it is percolated; there is no lag phase in the process. It has been found that nitrification can proceed to some measurable extent in these 'stimulated soils' before breakdown of any organic material added to the percolate causes serious immobilization of either ammonium or nitrate ions.

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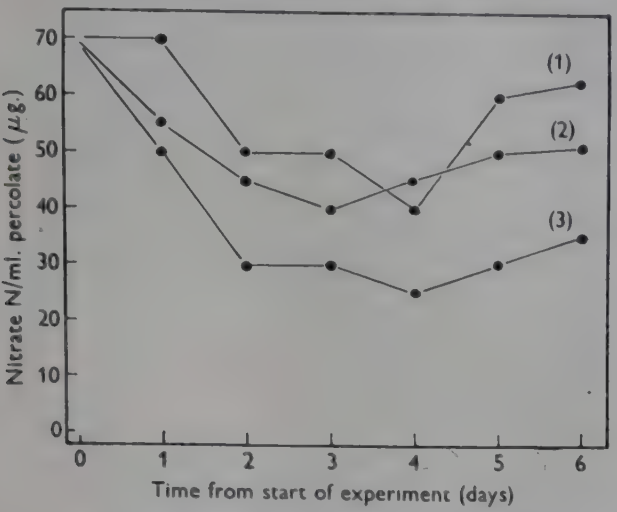

Fig. 1. The immobilization of nitrate N by organic materials percolated through allotment soil. Allotment soil (10 g.) percolated with 100 ml. 10^{-2}M-$NaNO_3$ containing: curve 1, sucrose; curve 2, glucose; curve 3, glycerol; all at 0·4 mg. C/ml.

The procedure used to test the effect of organic materials on soil nitrification was therefore as follows. Allotment soil in 10 g. lots was stimulated to active nitrification by percolation with 100 ml. of $2{\cdot}5 \times 10^{-3}$M-$(NH_4)_2SO_4$ for 12 days at 18–21°. The samples were then washed 3 times with 50 ml. lots of distilled water and finally washed by percolation with 100 ml. distilled water for 24 hr.; this percolate and the previous washings were discarded. All detectable nitrite and nitrate were thus removed from the soils, which were then percolated either with 100 ml. of $2{\cdot}5 \times 10^{-3}$M-$(NH_4)_2SO_4$ alone, or with 100 ml. of $2{\cdot}5 \times 10^{-3}$M-$(NH_4)_2SO_4$ + 0·1% of the organic material under test. Nitrite and nitrate formation by the soils in the presence and absence of organic material were determined by percolate analyses.

RESULTS

The first experiments were of 24 hr. duration. The formation of nitrite N and nitrate N during this period by soils percolated with ammonium sulphate alone and with ammonium sulphate plus glucose (or starch) are given in Table 1. Similar soils percolated with sodium nitrate, and with sodium nitrate plus 0·1% glucose (or starch) served as controls. The virtual absence of any nitrite formation by the control soils demonstrated that the appreciable quantities of nitrite formed by the test soils percolated with ammonium sulphate plus glucose (or starch) could not have arisen by reduction of traces of nitrate left in the soil. The nitrite formation observed in the presence of glucose and starch must have been from ammonium ions. This shows that in the allotment soil the oxidation of ammonium ions to nitrite ions, i.e. the first stage of nitrification, can proceed in the presence of 0·1% glucose or 0·1% starch. Nevertheless, before the 24 hr. were complete the glucose, but not the starch, percolates were showing a cloudiness, indicative of some heterotrophic proliferation that might have accounted for the obviously diminished nitrate-N formation by the glucose percolated soils. The next experiments, performed on a wider range of organic materials, were therefore of only 5 hr. duration (Table 2).

The results in Table 2 are complicated by breakdown of gelatin and peptone, but they agreed with those of Table 1 in showing that the organic materials tested had little effect on soil nitrification. This was checked by an experiment in which only 50 ml. of percolate were used (Table 3), and in which the concentration of nitrate N was reduced from 70 μg. NO_3-N/ml. to 10 μg. NO_3-N/ml. The results obtained agreed satisfactorily with those of Table 2, not only qualitatively but quantitatively. The quantitative agreement indicates that the estimation methods used for nitrite N and nitrate N were reliable, even though the total quantity of nitrogen estimated was only a few μg./ml.

Table 1. *The formation of nitrite* N *and nitrate* N *by* 10 *g. stimulated allotment soil in the presence of glucose and starch*

(Duration of experiment, 24 hr.)

	Nitrite N or nitrate N formed/g. soil (μg.)		
Soil percolated with 100 ml. of	Nitrite N	Nitrate N	Nitrite N + nitrate N
$2{\cdot}5 \times 10^{-3}$M-$(NH_4)_2SO_4$	20	220	240
$2{\cdot}5 \times 10^{-3}$M-$(NH_4)_2SO_4$ with 0·1% glucose	15	130	145
$2{\cdot}5 \times 10^{-3}$M-$(NH_4)_2SO_4$ with 0·1% starch	12	200	212
5×10^{-3}M-$NaNO_3$	0·1	—	—
5×10^{-3}M-$NaNO_3$ with 0·1% glucose	0·1	—	—
5×10^{-3}M-$NaNO_3$ with 0·1% starch	0·1	—	—

Table 2. *The formation of nitrite* N *and nitrate* N *by* 10 *g. stimulated allotment soil in the presence of various organic materials*

(Duration of experiment, 5 hr.)

Soil percolated with 100 ml. of	Nitrite N or nitrate N formed/g. soil (μg.)		
	Nitrite N	Nitrate N	Nitrite N + nitrate N
$2{\cdot}5\times10^{-3}$ M-$(NH_4)_2SO_4$	11, 16	31, 20	42, 36
$2{\cdot}5\times10^{-3}$ M-$(NH_4)_2SO_4$ and 0·1 % glucose	7, 15	32, 20	39, 35
$2{\cdot}5\times10^{-3}$ M-$(NH_4)_2SO_4$ and 0·1 % agar	7, 13	31, 19	38, 32
$2{\cdot}5\times10^{-3}$ M-$(NH_4)_2SO_4$ and 0·1 % gelatin	17	18	35
$2{\cdot}5\times10^{-3}$ M-$(NH_4)_2SO_4$ and 0·1 % peptone	31, 13	0, 22	31, 35
$2{\cdot}5\times10^{-3}$ M-$(NH_4)_2SO_4$ and 0·1 % humus	16	14	30
5×10^{-3} M-$NaNO_3$ and 0·1 % glucose	0·1, 0·1	—	—
5×10^{-3} M-$NaNO_3$ and 0·1 % agar	0·1, 0·1	—	—
5×10^{-3} M-$NaNO_3$ and 0·1 % gelatin	5	—	—
5×10^{-3} M-$NaNO_3$ and 0·1 % peptone	22, 5	—	—
5×10^{-3} M-$NaNO_3$ and 0·1 % humus	0·1	—	—
Water alone	0·1	c. 1	c. 1

Table 3. *The formation of nitrite* N *and nitrate* N *by* 10 *g. stimulated allotment soil in the presence of various organic materials*

(Duration of experiment, 5 hr.)

Soil percolated with 50 ml. of	Nitrite N or nitrate N formed/g. soil (μg.)		
	Nitrite N	Nitrate N	Nitrite N + nitrate N
$2{\cdot}5\times10^{-3}$ M-$(NH_4)_2SO_4$	22	14	36
$2{\cdot}5\times10^{-3}$ M-$(NH_4)_2SO_4$ and 0·1 % glucose	22	15	37
$2{\cdot}5\times10^{-3}$ M-$(NH_4)_2SO_4$ and 0·1 % agar	22	8	30
$2{\cdot}5\times10^{-3}$ M-$(NH_4)_2SO_4$ and 0·1 % gelatin	18	12	30
$2{\cdot}5\times10^{-3}$ M-$(NH_4)_2SO_4$ and 0·1 % peptone	26	4	30
$2{\cdot}5\times10^{-3}$ M-$(NH_4)_2SO_4$ and 0·1 % humus	20	10	30
5×10^{-4} M-$NaNO_3$	0	0	0
5×10^{-4} M-$NaNO_3$ and 0·1 % glucose	0	0	0
5×10^{-4} M-$NaNO_3$ and 0·1 % agar	0	0	0
5×10^{-4} M-$NaNO_3$ and 0·1 % gelatin	8	3	11
5×10^{-4} M-$NaNO_3$ and 0·1 % peptone	11	3	14
5×10^{-4} M-$NaNO_3$ and 0·1 % humus	0	0	0

DISCUSSION

The results reported show that if the organic materials that are supposed to inhibit nitrification in culture do so in soil, the inhibitions by 0·1 % of the materials tested are small. Moreover, if the lack of inhibition is due to a symbiosis between heterotrophs and nitrifiers, the symbiosis is either established with startling rapidity, or exists preformed in a soil not previously treated with organic material under test. Although results show reasonably clearly that 0·1 % gelatin has little effect on nitrification in soil, this does not mean that gelatin could have no effect on nitrification in culture at a similar concentration. Gelatins always contain 5–50 p.p.m. of lead, zinc and copper, and the actual balance of at least the last two of these trace elements may profoundly affect the speed of nitrification in culture (Meiklejohn & Lees, 1948). If, however, the reported inhibitions of culture nitrification were in fact due to trace-element disturbances, there is no possibility of demonstrating the same effect in a normal soil because in it the trace-element balance is strongly buffered by soil-complex reserves of these elements.

The present work seems to contradict much published work on the inhibition of culture nitrification by organic materials, but it should be remembered that the inhibitions observed by Winogradsky & Omeliansky (1899, 1902) and other subsequent workers may have been inhibitions not of nitrification, but of the growth of a young, and therefore sensitive, culture of nitrifying bacteria. Most of the experiments on the action of organic materials on culture nitrification have consisted in inoculating a sterile medium containing the organic material with a small amount of actively nitrifying culture, and following the subsequent formation, in the medium, of nitrite and nitrate. It is clear that any inhibition observed by this technique is as likely to be due to bacteriostasis as to inhibition of the characteristic activities of the bacteria. Meyerhof (1916 *a*, *b*, 1917) recognized the distinction, but unfortunately his results have not been given the attention they deserve. It should be mentioned however that Nelson (1931) evaluated the glucose inhibition of both nitrite formation and nitrate formation in culture by adding glucose to actively nitrifying cultures, and found that 10% glucose inhibited the nitrification by only 4%. It is precisely this method of evaluating inhibition of nitrification, which seems the only valid one, that has been used in the present work.

SUMMARY

1. The effects of various organic materials on the oxidative nitrification process in soil have been tested by a new short-term technique.

2. None of the materials, all of which were tested at 0·1% concentration, markedly inhibited the process.

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The Immobilization of Mineral Nitrogen in Soils by Different Organic Materials

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When an organic carbon source, such as glucose, is percolated through soil together with a source of mineral N, such as nitrate, a heterotrophic population quickly builds up at the expense of both compounds, and the amount of mineral N in the percolate decreases. When the supply of one compound is used up, the heterotrophic proliferation stops and the population begins to decline. The lysis of the dead cells produces ammonium ions, which are nitrified by the soil to nitrate ions, and the nitrate-N concentration begins to rise again. Not all the N initially immobilized is released again, because a certain amount is lost, as a result of its conversion to gaseous nitrogen by the denitrifying bacteria, some of which can function under aerobic conditions (Meiklejohn, 1940).

This nitrogen immobilization cycle in soil has often been studied in pots or in the field, but there seems to have been no detailed study of the biochemical processes involved. The work here reported is a preliminary survey of these processes.

METHODS

The soil percolation technique (Lees & Quastel, 1944) has been used throughout.

Soils were percolated with either $NaNO_3$ solution alone, or with $NaNO_3$ solution plus the organic material under test, in the original soil percolation apparatus (Lees, 1946*a*). Ammonium, nitrite, and nitrate N were determined from time to time in 2 ml. samples of the percolates by the analytical methods already devised (Lees & Quastel, 1946). In fact the nitrite-N and ammonium-N concentrations in the percolates were never found to be more than a few μg./ml., and for simplicity they are excluded from the reported results. When it was necessary to use CO_2-free air, the modified percolation apparatus (Lees, 1946*a*) was employed, and air was supplied to it through two large KOH bubblers followed by a Sofnolite indicator tube. When extra CO_2 was necessary, it was introduced into the reservoir by means of a bubbler tube coupled to a Microid CO_2 generator. The CO_2 was supplied at the rate of approximately 1 bubble of CO_2/10 bubbles of entrant air.

Two soils have been used: (1) The garden soil (0·4% organic N, pH 7·8) used by Lees & Quastel (1946) (this soil is here described as the allotment soil); (2) Romney Marsh soil (0·2% organic N, pH 8·0) used by Lees (1946*b*).

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RESULTS

The results of investigations on nine different organic compounds, percolated through allotment soils, are given in Table 1. The pH of the percolates rose about 0·5 during the early stages of the experiment because the immobilization of the anionic NO_3' released cationic Na^+. In the later stages the pH again fell to normal as NO_3' was generated. It will be seen that the initial nitrogen immobilization differed for different organic compounds. The final concentration of nitrate N, eventually built up in the percolate by lysis of the dead heterotrophs, was always less than the nitrate concentration of the nitrate-control percolate. The difference between the nitrate concentration of the control percolate and the nitrate concentration of the test percolate was presumably (at least towards the end of an experiment) some measure of denitrification loss. This loss also differed for different organic compounds. The constancy of the nitrate-N concentration of the control percolates indicated that there was no endogenous-nitrogen immobilization by compounds in the soil itself.

Table 1. *The immobilization of nitrate* N *by different organic compounds*

(Soil: allotment, 40 g. Percolate: 200 ml. 10^{-2} M-$NaNO_3$ plus the organic compound at 1·2 mg. C/ml. The minimum nitrate-N concentrations are italicized.)

Organic compound added	Nitrate N (µg./ml. percolate) after 4 days	8 days	12 days	20 days	24 days
None (control)	150	151	158	150	151
Glycerol	*8*	12	35	56	57
Glucose	*17*	*20*	80	93	104
Sucrose	*23*	26	81	86	103
K oleate	*20*	25	69	47	61
K stearate	*12*	20	32	50	106
None (control)	140	140	140	141	—
Na formate	102	*79*	116	135	—
Tristearin	135	130	134	*129*	—
Tributyrin	*19*	50	68	79	—
Benzoic acid	70	*60*	77	125	—

The magnitudes of the temporary immobilization of mineral nitrogen, and eventual loss of total nitrogen, are not however solely dependent on the type of carbon source. Both increase as the amount of soil in the soil-percolate system is decreased. Table 2 shows the results from an experiment in which various weights of a Romney Marsh soil were percolated with 200 ml. of 10^{-2} M-$NaNO_3$ plus glucose at 0·8 mg. C/ml. Marsh soil has good buffering properties, and no marked pH changes of the percolate were noted during the experiment. The same type of result has been obtained with allotment soil (Fig. 1) in the new percolation apparatus (Lees, 1947).

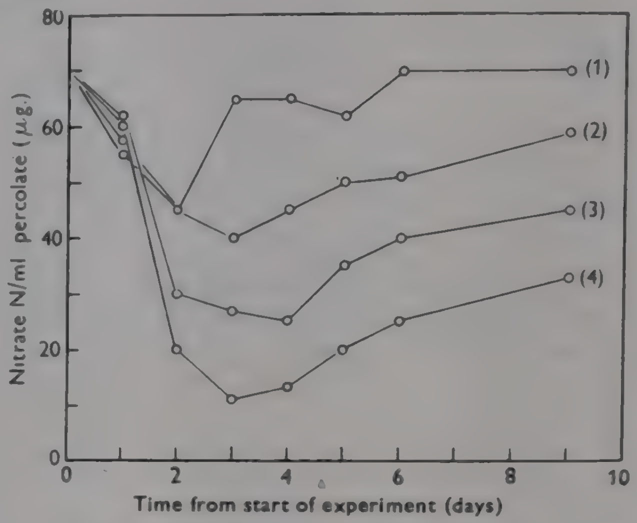

Fig. 1. The immobilization of nitrate N by various weights of allotment soil percolated with 100 ml. 10^{-2} M-$NaNO_3$ plus glucose at 0·4 mg. C/ml. Curve 1, 20 g. soil; curve 2, 10 g. soil; curve 3, 5 g. soil; curve 4, 2·5 g. soil.

The amount of nitrogen immobilized by a given amount of any one organic material seems to depend to some extent on the amount of free carbon dioxide in the soil percolate system (Table 3). Although the removal of carbon dioxide from the air entering the apparatus made no difference to the amount of nitrogen immobilized by a given amount of any one organic material (presumably because small amounts of carbon dioxide were in any case generated by the metabolic processes of the soil), the addition of carbon dioxide to the air entering the apparatus increased the amount of nitrogen immobilized.

A final point of interest is that the nitrogen-immobilizing mechanism appears to be poisoned by 10^{-3} M-azide, but not by similar concentrations of either iodoacetate or chlorate (Table 4). Un-

Table 2. *The effect of the change of soil weight on nitrogen immobilization and nitrogen loss in Romney Marsh soil percolated with nitrate and glucose*

(Percolate: 200 ml. 10^{-2} M-$NaNO_3$ + glucose at 0·8 mg. C/ml.)

Weight of soil (g.)	Nitrate N (µg./ml. percolate) after 1 day	2 days	3 days	4 days	5 days	6 days	9 days	13 days	16 days
2	121	92	97	100	90	71	52	32	43
10	130	114	83	65	68	49	63	59	77
20	138	95	82	75	87	85	87	91	107
40	134	94	80	80	112	108	126	126	144

Table 3. *The effect of* CO_2 *on nitrogen immobilization*

(Weight of soil: 40 g. Percolate: 200 ml. 10^{-2} M-$NaNO_3$ plus organic material at approx. 1·0 mg. C/ml.)

Soil	Organic material	Air supply	Nitrate N immobilized/mg. carbon supplied (μg.)
Allotment	None	CO_2-free + 10 % CO_2	0
Allotment	Glucose	Ordinary	110
Allotment	Glucose	CO_2-free	99
Allotment	Glucose	CO_2-free + 10 % CO_2	134
Allotment	Lactic acid	Ordinary	108
Allotment	Lactic acid	CO_2-free	103
Allotment	Lactic acid	CO_2-free + 10 % CO_2	143
Marsh	None	CO_2-free + 10 % CO_2	0
Marsh	Lactic acid	Ordinary	67
Marsh	Lactic acid	CO_2-free	70, 100, 69
Marsh	Lactic acid	CO_2-free + 10 % CO_2	104, 143, 88
Marsh	Malic acid	CO_2-free	67, 48
Marsh	Malic acid	CO_2-free + 10 % CO_2	100, 67
Marsh	Fumaric acid	CO_2-free	61, 51
Marsh	Fumaric acid	CO_2-free + 10 % CO_2	67, 65
Marsh	Succinic acid	CO_2-free	57
Marsh	Succinic acid	CO_2-free + 10 % CO_2	69

Table 4. *The effects of sodium azide, iodoacetate, and chlorate on nitrogen immobilization in allotment soil*

(Soil (40 g.) was percolated with 200 ml. 10^{-2} M-$NaNO_3$ with glucose (equivalent to 1·2 mg. C/ml.) and 10^{-3} M-azide, iodoacetate or chlorate. Control soil percolated with nitrate and glucose only. Negative control soil percolated with nitrate only.)

	Nitrate N (μg./ml. percolate) after					
Poison added to percolate	1 day	4 days	6 days	8 days	12 days	15 days
Na azide	144	145	134	101	115	119
Na iodoacetate	107	0	15	10	45	32
Na chlorate	140	16	30	26	54	63
Control soil	110	24	50	56	72	76
Negative control soil	158	160	156	159	159	153

published results obtained by Dr J. H. Quastel and myself have suggested that 10^{-3} M-iodoacetate or azide is toxic to nitrification in soil. Results showing the effect of 10^{-3} M-chlorate on soil nitrification have already been published (Lees & Quastel, 1945).

DISCUSSION

The results are preliminary but suggest that the immobilization of soil nitrogen by various organic materials can be studied quantitatively by a soil percolation technique.

Both nitrogen immobilization and denitrification loss have been found to vary with the type of organic compound used to bring them about. Moreover, both increase as the percolate : soil ratio is increased. Lemmermann & Wickers (1914) found, by pot experiments, that denitrification in soil was increased as the fluid : soil ratio was increased. It is possible to explain these results on the assumption that a high water content in a soil favours anaerobiosis, and therefore favours denitrification; it is not possible similarly to explain the present results. In the percolation apparatus denitrification and nitrogen immobilization were found to be greatest where the smallest weight, i.e. the shortest column, of soil was used; in other words, they were found to be greatest where anaerobiosis must have been minimal. One theory that might explain this result is that proliferation of the heterotrophs, denitrifying and other, takes place mainly in the percolate while lysis of the same heterotrophs is mainly accomplished by organisms or other agencies closely bound to the soil surface.

There are reasons for believing that nitrogen immobilization in soil may involve heavy-metal enzymes because the process is poisoned by azide. Either sulphydryl enzymes do not play any important part in nitrogen immobilization, or iodoacetate is rapidly destroyed in the soil under the experimental conditions, because iodoacetate has, if anything, a stimulatory effect on nitrogen immobilization. The chlorate-sensitive enzyme system that was found in the nitrifying bacteria by Lees & Quastel (1945) does not appear to be involved.

The effect of CO_2 in increasing nitrogen immobilization in soil is interesting because, if the increase is due to CO_2 as CO_2, and not simply to a lowering of the pH at the soil surface (where micro-organisms in general congregate), then CO_2 must be considered as one possible source of organic carbon in soil. The work of Krampitz, Woods & Werkman (1943) is germane to this point.

It is unfortunately not possible for me to continue this work at the moment. The results are presented as provisional ones that might provide some useful pointers in future investigations on the biochemistry of nitrogen immobilization by soil.

SUMMARY

1. The immobilization of nitrogen in soils by various organic materials has been studied by a soil-percolation technique.

2. The amount of nitrogen immobilization, and the amount of denitrification, induced by an organic compound differs from compound to compound.

3. The effects of enzyme poisons and of carbon dioxide on the immobilization process suggest that some heavy-metal enzyme and some carbon dioxide-assimilative process are involved.

Most of this work was done at the Agricultural Research Council Unit of Soil Metabolism. I should like to acknowledge help and advice from Dr J. H. Quastel and to thank the A.R.C. for permission to publish these results.

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The Effects of Zinc and Copper on Soil Nitrification

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(*Received 22 September* 1947)

Although many papers have been published dealing with the effects of trace elements on plants, and on soil processes in the field, there appears to have been no exhaustive study of the effect of trace elements on the microbial processes of the soil. As these processes are an essential part of agriculture, the effect of trace elements is of practical as well as theoretical interest. Hence the work here reported was begun.

METHODS

The soil-percolation technique devised by Lees & Quastel (1946*a*) for the study of soil metabolism has been used throughout. The soil-percolation apparatus used was the simplified and improved form (Lees, 1947). The influence of either zinc or copper on soil nitrification was assessed by comparing the course of nitrification in a soil percolated with $(NH_4)_2SO_4$ solution with the course of nitrification in a soil percolated with a similar $(NH_4)_2SO_4$ solution to which a known concentration of either $ZnSO_4$ or $CuSO_4$ had been added.

Mineral nitrogen analyses were performed from time to time on samples of the percolates by methods similar to those already described (Lees & Quastel, 1946*a*). As it was found early in the work that the conversion of ammonium N into nitrate N was always reasonably quantitative, ammonium-N estimations are not included in the results. Nitrite N was never found in quantities greater than a few μg./ml.; nitrite N is therefore bulked with nitrate in the reported nitrate-N figures. Percolate analyses have been converted into 'μg. N/g. soil' by the methods already described (Lees & Quastel, 1946*a*).

Copper was estimated in the percolates by the method of Sherman & McHargue (1942). Zinc was estimated spectrophotometrically.

Soils were stored damp in earthenware containers and turned from time to time. Samples of soil were taken from these containers, and air-dried and sieved (4·0–1·0 mm. mesh) before use.

All experiments were conducted with 10 g. soil percolated at 18–21° with 100 ml. of $2\cdot5 \times 10^{-3}$M-$(NH_4)_2SO_4$, with or without the addition of $ZnSO_4$ or $CuSO_4$. Three soils were used: (1) an allotment soil (0·4% organic N, pH 6·8); (2) a Lincolnshire Fen soil (2·5% organic N, pH 7·0); (3) a Romney Marsh soil (0·2% organic N, pH 7·8).

Stimulated soils (Lees & Quastel, 1946*b*) were prepared as follows. The soils were first percolated with 100 ml. of $2\cdot5 \times 10^{-3}$M-$(NH_4)_2SO_4$ until nitrification was complete, washed out two or three times with 50 ml. lots of distilled water, and finally percolated with 100 ml. distilled water for 6 hr. This wash percolate was then discarded and the soils were ready for the experiment proper.

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Poisoning of a soil by diethyldithiocarbamate was carried out as follows. Ten ml. of 4×10^{-3} M-sodium diethyldithiocarbamate were poured on to the stimulated but unwashed soil. The liquid that dripped through was recovered and again poured on to the soil. This process was thrice repeated in order to distribute the poison evenly through the soil, which was then left untouched for 1 hr. Excess of the poison was removed from the soil by three 50 ml. washings with distilled water followed by percolation for 6 hr. with 100 ml. distilled water. This wash percolate was then discarded and the soil was ready for the experiment.

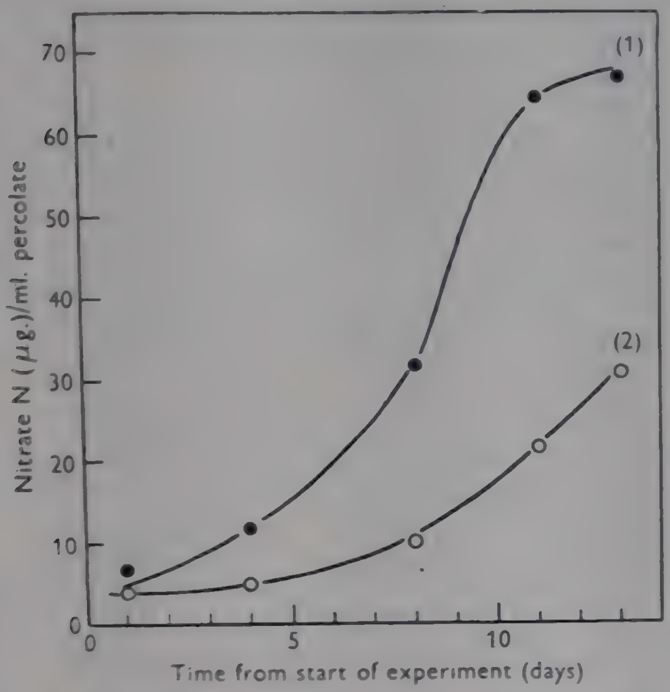

Fig. 1. The effect of zinc on nitrification in fresh (unstimulated) allotment soil. Soil (10 g.) was percolated with 100 ml. of (curve 1) $2 \cdot 5 \times 10^{-3}$M-$(NH_4)_2SO_4$ or (curve 2) $2 \cdot 5 \times 10^{-3}$M-$(NH_4)_2SO_4$ plus 4×10^{-3}M-$ZnSO_4$.

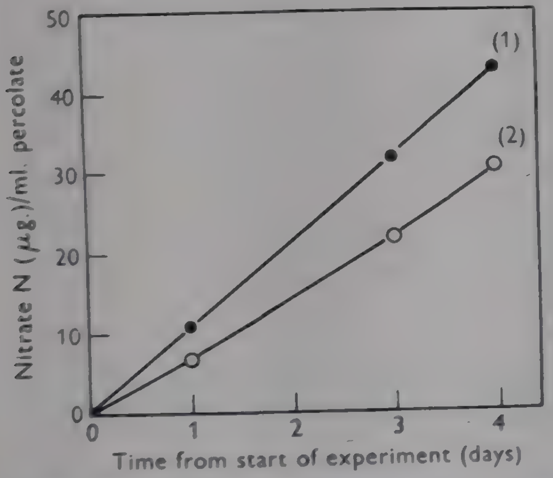

Fig. 2. The effect of zinc on nitrification in stimulated allotment soil. Soil (10 g.) was percolated with 100 ml. of (curve 1) $2 \cdot 5 \times 10^{-3}$M-$(NH_4)_2SO_4$ or (curve 2) $2 \cdot 5 \times 10^{-3}$M-$(NH_4)_2SO_4$ plus 4×10^{-3}M-$ZnSO_4$.

Experimental procedure. The toxic effect of zinc on soil nitrification may be demonstrated either as a flattening of the sigmoid curve characteristic of nitrification in a fresh soil (Fig. 1), or as a reduction of the slope of the straight line characteristic of nitrification in a stimulated soil (Fig. 2).

The inhibition due to zinc in either case may be measured as

$$100 \times \frac{C-T}{T},$$

where C = nitrate formation in absence of zinc, and T = nitrate formation in presence of zinc. This value would be constant throughout the control nitrification in ideal experiments with stimulated soils (Fig. 2), but it is less obviously so in experiments with unstimulated soils (Fig. 1).

In experiments with unstimulated soils the inhibition of nitrification due to zinc has therefore been measured at two roughly defined points during the experiment: (1) when control nitrification was half completed, and (2) when control nitrification was 90% completed. In practice the inhibition measured at point (1) has been found to agree well with that measured at point (2). But because the use of stimulated soils excludes spurious effects of zinc on the initial proliferation rate of the nitrifying organisms and minimizes the possibility of their adapting their activities to the presence of zinc, stimulated soils rather than unstimulated soils have been used in most of the work reported here.

The above explanation is based on the effect of zinc on soil nitrification; it should be understood to apply equally well to the effect of copper on the same process.

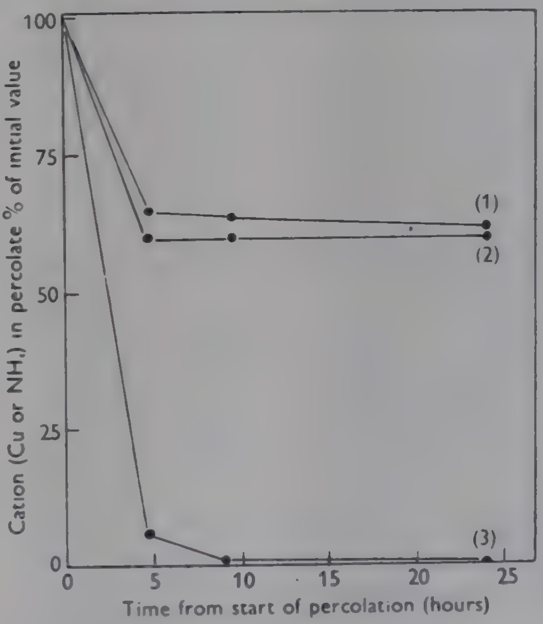

Fig. 3. The removal of ammonium and copper ions from the percolates of 10 g. Marsh soil percolated with 100 ml. of either 5×10^{-3}M-$(NH_4)_2SO_4$ or 5×10^{-3}M-$(NH_4)_2SO_4$ plus 5×10^{-3}M-$CuSO_4$. Curve 1, ammonium ion concentration in presence of copper; curve 2, ammonium ion concentration in absence of copper; curve 3, copper ion concentration.

RESULTS

When a solution containing zinc or copper is percolated through a soil, nearly all the zinc or copper is, within a few hours, transferred from the percolate to the soil. Fig. 3 illustrates the removal of copper from copper sulphate percolated through Marsh soil, but it should be understood to typify the result obtained when a solution of either zinc or copper is percolated through any soil. The mechanism responsible for the transfer of zinc or copper from percolate to soil is not the normal soil base-exchange system; the normal base-exchange equilibrium reached by ammonium ions percolated at the same time through the same soil is unaffected by the concomitant transfer of zinc or copper to the soil. The zinc and copper are probably taken up by the organic matter of the soil (Bremner, Mann, Heintze & Lees, 1946; Brun, 1945); therefore the percolate concentrations of zinc and copper given in the tables are initial ones. Within 24 hr. from the start of any experiment reported the percolate concentration of either zinc or copper was certainly less than 0·5 μg./ml., but no exact determination of this value was carried out.

Complete protocols of experiments on the effects of zinc and copper on nitrification in unstimulated Romney Marsh soil are given in Table 1. The results from parallel experiments with stimulated Romney Marsh soil are given in Table 2.

As these two tables adequately illustrate the type of experimental data obtained, the results from experiments on stimulated allotment soil (Table 3) and stimulated Fen soil (Table 4) are given simply as mean values from replicate experiments. The results given in Tables 1–4 agree in showing that zinc was more inhibitory of soil nitrification than was copper. Low concentrations of copper appeared

Table 1. *The effects of zinc and copper sulphates on nitrification in unstimulated Romney Marsh soil*

(Soil (10 g.) was percolated with 100 ml. $2{\cdot}5\times10^{-3}$ M-$(NH_4)_2SO_4$, with or without $ZnSO_4$ or $CuSO_4$. Results are given (1) at half completion of control nitrification; (2) at 90 % completion of control nitrification.)

Metal in percolate (M)	Nitrate N formed/g. soil in different experiments (μg.)							Inhibition of nitrification (%)
(1) Nil	260	280	300	320	330	330	370	—
$ZnSO_4$, 4×10^{-3}	—	—	0	90	110	—	150	74
$ZnSO_4$, 2×10^{-3}	250	—	200	250	160	160	100	41
$ZnSO_4$, 1×10^{-3}	110	230	250	170	320	200	230	31
$ZnSO_4$, 5×10^{-4}	—	280	300	300	—	—	—	2
Nil	390	370	340	340	—	—	—	—
$CuSO_4$, 4×10^{-3}	260	160	200	—	—	—	—	44
$CuSO_4$, 2×10^{-3}	—	260	260	200	—	—	—	31
$CuSO_4$, 1×10^{-3}	390	350	250	260	—	—	—	13
$CuSO_4$, 5×10^{-4}	400	350	—	—	—	—	—	1
(2) Nil	670	590	600	700	700	700	700	—
$ZnSO_4$, 4×10^{-3}	—	—	100	240	160	—	350	69
$ZnSO_4$, 2×10^{-3}	500	—	550	440	500	360	270	36
$ZnSO_4$, 1×10^{-3}	160	530	500	700	680	690	550	18
$ZnSO_4$, 5×10^{-4}	—	590	600	700	—	—	—	0
Nil	650	730	690	690	—	—	—	—
$CuSO_4$, 4×10^{-3}	550	440	350	—	—	—	—	35
$CuSO_4$, 2×10^{-3}	—	600	570	500	—	—	—	21
$CuSO_4$, 1×10^{-3}	650	680	690	700	—	—	—	1
$CuSO_4$, 5×10^{-4}	670	710	—	—	—	—	—	0

Table 2. *The effects of zinc and copper sulphates on nitrification in stimulated Romney Marsh soil*

(Soil (10 g.) was percolated with 100 ml. $2{\cdot}5\times10^{-3}$ M-$(NH_4)_2SO_4$, with or without $ZnSO_4$ or $CuSO_4$. A negative inhibition means that nitrification was stimulated.)

Metal in percolate (M)	Nitrate N formed/g. soil in different experiments after		Inhibition of nitrification after	
	3 days (μg.)	5 days (μg.)	3 days (%)	5 days (%)
Nil	250, 270, 520, 500, 310, 310	730, 680, 700, 720, 710, 710	—	—
$ZnSO_4$, 4×10^{-3}	0, 30, 0, 100, 30, 0	0, 140, 150, 200, 0, 0	93	88
$ZnSO_4$, 2×10^{-3}	170, 140, 400, 150, 200, 140	430, 450, 500, 580, 340, 370	44	37
$ZnSO_4$, 1×10^{-3}	120, 70, 250, 370, 140, 240	400, 200, 470, 700, 580, 450	45	34
$ZnSO_4$, 5×10^{-4}	220, 240, 300, 350, 310, 300	580. 670, 720, 700, 600, 470	20	12
Nil	290, 270, 430, 410	750, 680, 600, 690	—	—
$CuSO_4$, 4×10^{-3}	250, 160, 270, 270	300, 300, 370, 440	32	48
$CuSO_4$, 2×10^{-3}	290, 140, 370, 490	740, 550, 450, 600	8	14
$CuSO_4$, 1×10^{-3}	320, 270, 410, 400	750, 620, 580, 630	0	5
$CuSO_4$, 5×10^{-4}	250, 340, 370, 480	740, 670, 590, 700	−3	1

Table 3. *The effects of zinc and copper sulphates on nitrification in stimulated allotment soil*

(Soil (10 g.) was percolated with 100 ml. $2\cdot5 \times 10^{-3}$ M-$(NH_4)_2SO_4$, with or without $ZnSO_4$ or $CuSO_4$. Each figure is the mean value from four separate experiments. A negative inhibition means that nitrification was stimulated.)

Metal in percolate (M)	Nitrate N formed/g. soil after			Mean inhibition of nitrification after		
	1 day (µg.)	3 days (µg.)	4 days (µg.)	1 day (%)	3 days (%)	4 days (%)
Nil	120	320	440	—	—	—
$ZnSO_4$, 4×10^{-3}	40	40	90	67	87	80
$ZnSO_4$, 2×10^{-3}	80	210	270	33	34	39
$ZnSO_4$, 1×10^{-3}	80	250	320	33	22	27
$ZnSO_4$, 5×10^{-4}	95	250	350	21	22	21
$ZnSO_4$, $2\cdot5 \times 10^{-4}$	110	250	405	8	22	8
Nil	120	340	450	—	—	—
$CuSO_4$, 4×10^{-3}	30	80	140	75	76	69
$CuSO_4$, 2×10^{-3}	75	260	360	37	24	20
$CuSO_4$, 1×10^{-3}	100	270	380	17	21	16
$CuSO_4$, 5×10^{-4}	160	310	435	−33	9	3
$CuSO_4$, $2\cdot5 \times 10^{-4}$	140	370	470	−17	−9	−4

to stimulate nitrification a little, but the stimulation was not significant. Nevertheless the implied suggestion that copper may play some part in soil nitrification is supported by the results of experiments in which Fen soils were treated with sodium diethyldithiocarbamate (Table 5). This treatment, which removed 13 µg. Cu/g. soil (about 30 times the exchangeable copper of the soil), effectively inhibited soil nitrification. The inhibition could be reversed completely by percolating copper sulphate through the soils, partially by percolating manganese sulphate, but not at all by percolating ferrous sulphate, zinc sulphate, or cadmium chloride.

Table 4. *The effects of zinc and copper sulphates on nitrification in stimulated Fen soil*

(Soil (10 g.) was percolated with 100 ml. $2\cdot5 \times 10^{-3}$ M-$(NH_4)_2SO_4$, with or without $ZnSO_4$ or $CuSO_4$. Each figure is the mean value from three separate experiments. A negative inhibition means that nitrification was stimulated.)

Metal in percolate (M)	Nitrate N formed/g. soil after		Mean inhibition of nitrification after	
	2 days (µg.)	4 days (µg.)	2 days (%)	4 days (%)
Nil	240	520	—	—
$ZnSO_4$, 4×10^{-3}	150	410	37	21
$ZnSO_4$, 2×10^{-3}	180	340	25	35
$ZnSO_4$, 1×10^{-3}	180	500	25	4
$ZnSO_4$, 5×10^{-4}	230	430	4	17
$ZnSO_4$, $2\cdot5 \times 10^{-4}$	220	510	8	2
Nil	230	420	—	—
$CuSO_4$, 4×10^{-3}	230	340	0	19
$CuSO_4$, 2×10^{-3}	200	360	13	14
$CuSO_4$, 1×10^{-3}	230	400	0	5
$CuSO_4$, 5×10^{-4}	220	440	4	−5
$CuSO_4$, $2\cdot5 \times 10^{-4}$	240	440	−4	−5

Table 5. *The effects of various metals in reversing diethyldithiocarbamate poisoning of nitrification in Fen soil*

(Diethyldithiocarbamate-treated soils (10 g.) were percolated with 100 ml. $2\cdot5 \times 10^{-3}$ M-$(NH_4)_2SO_4$ plus metals as indicated. Control soil was not treated with diethyldithiocarbamate.)

Metal addition to percolate (M)	Nitrate N formed/g. soil after		
	1 day (µg.)	3 days (µg.)	5 days (µg.)
Nil (control)	160	350	620
Nil	30	80	130
$CuSO_4$, 4×10^{-3}	110	360	490
$CuSO_4$, 2×10^{-3}	100	300	580
$CuSO_4$, 1×10^{-3}	80	320	580
$CuSO_4$, 5×10^{-4}	40	250	590
$ZnSO_4$, 4×10^{-3}	30	30	100
$ZnSO_4$, 2×10^{-3}	20	60	100
$CdCl_2$, 4×10^{-3}	30	40	110
$CdCl_2$, 2×10^{-3}	30	60	110
$FeSO_4$, 4×10^{-3}	20	40	140
$FeSO_4$, 2×10^{-3}	30	80	150
$MnSO_4$, 4×10^{-3}	20	120	210
$MnSO_4$, 2×10^{-3}	30	120	200

DISCUSSION

The results indicate that it should be possible to study quantitatively by the percolation technique the effect of any trace element on the microfloral activities of soil. Zinc has been found to be more inhibitory to soil nitrification than copper, but the inhibition due to either metal is less on an organic soil than on a mineral soil. This accords with the agronomic finding that trace-element deficiencies are more usually associated with organic, rather than mineral, soils. In an organic soil the availability of a trace element (and therefore its toxicity, if it is toxic) tends to be less than in a mineral soil.

It would be unwise to conclude either from the present, or from the previous results (Lees, 1946), that copper is essential to the nitrifying organisms. The balance, as well as the amounts, of trace elements in a nitrifying culture may profoundly affect the speed of nitrification therein (Meiklejohn & Lees, in preparation). The inhibition of soil nitrification that ensues when copper is removed from a soil may therefore be due, not directly to a copper deficiency, but to a resultant imbalance of the remaining available trace elements. Such a theory would perhaps explain the partial reversal of diethyldithiocarbamate poisoning by manganese. Lucas (1945) has shown by plant growth experiments that there is some interaction between soil copper and soil manganese.

The results of the present work suggest a possible basis for a direct biological test for trace element deficiencies in soil. A diethyldithiocarbamate-treated soil is copper-deficient; as a result of this deficiency the nitrifying power of the soil is lowered, but may be raised again if copper is added to the soil. Copper deficiency in soil is therefore biologically detectable by the fact that addition of copper to the soil increases the nitrifying power. The possibility of measuring natural trace-element deficiencies by this sort of technique, which is an extension of the culture test elaborated by Mülder (1940), is obvious.

SUMMARY

1. It has been found possible quantitatively to study the effects of zinc and copper on soil nitrification by the percolation technique.

2. The toxic effect of either element is less on an organic than on a mineral soil.

3. Sodium diethyldithiocarbamate poisons soil nitrification. The poisoning is reversible by copper and partially reversible by manganese.

4. The possible importance of copper in nitrification is discussed.

5. It is suggested that the results might form the basis of a direct soil test for trace-element deficiencies.

I am indebted to Mr W. J. Redmond for accurate and skilled assistance throughout the work, and to Dr J. B. Hale for spectrophotometric determinations of zinc.

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Studies in Detoxication

16. THE METABOLISM OF ACETANILIDE IN THE RABBIT

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It is now sixty years since Cahn & Hepp (1887) introduced acetanilide (antifebrin) as an analgesic and antipyretic. Nevertheless, its fate in the body has not been studied quantitatively until recently (Greenberg & Lester, 1946). Furthermore, its metabolites are not exactly known. Early work (Müller, 1887; Kumagawa, 1888; Gregoire & Hendrick, 1904, etc.) established that acetanilide was oxidized *in vivo*, and that by suitable treatment of the urine *p*-aminophenol could be isolated. Jaffé & Hilbert (1888) found that the metabolites of acetanilide in the dog were different from those in the rabbit. From dog urine the main compound isolated was benzoxazolone together with small amounts of *p*-aminophenol, whereas from rabbit urine only *p*-aminophenol was isolated. It is likely that the benzoxazolone of Jaffé & Hilbert (1888) is an artefact derived from *o*-aminophenol and urea. It appears therefore that in dogs acetanilide is oxidized mainly in the *o*-position and in rabbits in the *p*-position. None of the above-mentioned work gives any clue whether acetanilide is deacetylated or not.

In 1889 Mörner, carefully avoiding hydrolytic procedures, worked up urine from a subject to whom acetanilide had been administered, and isolated potassium *p*-acetamidophenylsulphate as a double salt with potassium ethyl oxalate. He also isolated a glucuronide which he was unable to characterize, but thought to be p-acetamidophenylglucuronide. Mörner's work suggests that the metabolites of acetanilide in man are acetylated.

From a study of the pharmacological properties of acetanilide and a number of related compounds, Hinsberg & Treupel (1894) concluded that the antipyretic and analgesic properties of the acetanilide group of drugs were related to their conversion in the organism to p-aminophenol or p-acetamidophenol. They found both these phenols to have a prompt antipyretic action in man (p-acetamidophenol is a more powerful antipyretic than phenacetin), but the action was not prolonged enough for successful use in therapy. It therefore appeared that the activity of acetanilide or phenacetin could be explained by its slow conversion *in vivo* into one or other of these phenols. Hinsberg & Treupel (1894) favoured p-acetamidophenol. More recently, Michel, Bernheim & Bernheim (1937) have shown that acetanilide is split into aniline and acetic acid by rat liver and kidney tissue, and that aniline can be oxidized by the same tissues to p-aminophenol. From this it appears possible that acetanilide might be deacetylated and then oxidized to p-aminophenol.

The quantitative work of Greenberg & Lester (1946) on the fate of acetanilide in man has shown that 70–90 % of the dose is excreted as conjugated p-aminophenol, 96 % as O conjugates (i.e. glucuronide and ethereal sulphates) and 4 % as N conjugates (probably p-acetamidophenol). Other metabolites of acetanilide have been postulated by various workers, e.g. N-acetyl-N-phenylhydroxylamine (Ellinger, 1920) and certain azo compounds (Greenberg & Lester, 1946), but there appears to be no real basis for the existence of these metabolites.

From the work discussed it is therefore clear that the exact nature of the metabolites of acetanilide is not known. In the present paper evidence will be presented to show that, in the rabbit, the main metabolites are the glucuronide and ethereal sulphate of p-acetamidophenol. Other work in progress in this laboratory indicates that acetanilide and aniline are not metabolically equivalent as is sometimes thought, for the metabolites of aniline are not the same as those of acetanilide.

METHODS

Animals. Rabbits fed on 50 g. Lever's cubes daily were used throughout the work. Compounds were administered by stomach tube with water.

Glucuronic acid in urine was determined by the method of Hanson, Mills & Williams (1944).

Ethereal sulphate was determined by Folin's gravimetric method.

EXPERIMENTAL AND RESULTS

Table 1 indicates that the ratio glucuronide/ethereal sulphate (G/E) for acetanilide (5·8) is similar to that for p-acetamidophenol (6·3) and different from that for p-aminophenol (2·5). These figures could be interpreted as meaning that acetanilide is first oxidized to p-acetamidophenol which then conjugates with glucuronic and sulphuric acids in the same ratio as though the phenol itself were fed. The G/E ratio for p-aminophenol is so different from that of acetanilide that it could hardly be expected that acetanilide is transformed to p-aminophenol, i.e. via aniline. That this interpretation is correct is supported by the isolation experiments described below. The sum of the glucuronic acid and ethereal sulphate outputs ($G+E$) shows that 82 % of the acetanilide can be accounted for. $G+E$ is somewhat less for p-acetamidophenol (73 %) and for p-aminophenol (60·5 %). This lesser recovery of the phenols can be accounted for by the fact that they are rapidly absorbed and excreted and some appear in the free state in the urine. In the case of acetanilide there is a slow conversion to the phenol, which therefore becomes more completely conjugated. Here we have an illustration of the time factor in detoxication.

Table 1. *The output of glucuronic acid and ethereal sulphate in rabbits receiving orally acetanilide and related compounds*

Compound	Dose (g./kg.)	% of dose excreted as Glucuronide* (G)	Ethereal* sulphate (E)	$G+E$	G/E
Acetanilide	0·25	70	12	82	5·8
p-Acetamidophenol	0·28	63	10	73	6·3
p-Aminophenol	0·25	43	17·5	60·5	2·5

* Average results.

The effect of acetanilide on rabbits. In all experiments with rabbits receiving oral doses of acetanilide of 0·5 g./kg. it was observed that the animals fell into a deep narcosis for 4–5 hr. The animals all recovered without any apparent deleterious effect. This narcosis was not observed when the animals received p-acetamidophenol or p-aminophenol.

ISOLATION OF METABOLITES FROM ACETANILIDE URINE

(*a*) *Nature of the urine.* Urine from rabbits receiving oral doses of 0·5 g./kg. of acetanilide does not reduce Benedict's reagent except on prolonged boiling. It does not reduce cold ammoniacal $AgNO_3$. The urine does not give a colour with $FeCl_3$, nor does an ether extract of the untreated urine. The urine gives an intense naphthoresorcinol reaction for glucuronic acid and is laevorotatory.

(*b*) *Extraction at alkaline* pH. 2 g. of acetanilide were fed to each of 5 rabbits and the urine (1 l.) collected during the next 24 hr. A few ml. of 2N-KOH were added to the urine, which was then extracted continuously with ether for 1 hr. The extract on evaporation left a small oily residue which partly crystallized. A few drops of 2N-HCl were added to the residue and the whole was filtered, leaving a crystalline mass (40 mg.), which on recrystallization from hot water, was identified as acetanilide (m.p. and mixed m.p. 112–113°). The filtrate from these crystals was now made alkaline and extracted with ether. The ether was evaporated and the residue benzoylated with benzoyl chloride and NaOH. From the mixture there were isolated 20 mg. of benzanilide (m.p. and mixed m.p. 160°). The yields of aniline and acetanilide in four experiments are given in Table 2. Aminophenols were not found in this extract.

Table 2. *Recovery of acetanilide and aniline from the urine of rabbits receiving acetanilide orally*

	% of dose recovered as	
Exp.	Acetanilide	Aniline
1	0	0·5
2	0·4	0·1
3	0·2	0·1
4	0·2	0

(*c*) *Extraction at acid* pH. The above urine was made acid to congo red with HCl and extracted with ether for 1 hr. A small tarry residue remained on evaporation of the ether, but this contained nothing of significance.

(*d*) *Mild hydrolysis of acetanilide urine.* A total of 6 g. of acetanilide was fed to three rabbits and the urine (500 ml.) was collected during the following 24 hr. 350 ml. of the urine were made alkaline with a few ml. of 2N-KOH and then extracted continuously for 5 hr. with ether. Evaporation of the ether left 10 mg. of acetanilide; no aniline was found. The extracted urine was now made normal with respect to HCl by the addition of conc. HCl and then boiled gently for 20 min. A separate experiment showed that *p*-aminophenylglucuronide was only slightly hydrolyzed under these conditions. The urine was now cooled and made slightly alkaline with solid Na_2CO_3 and then extracted for 1 hr. with ether. On cooling the ether extract, 200 mg. of *p*-aminophenol separated (m.p. 184° after recrystallization). The ether was now taken to 5 ml. and a further 350 mg. of *p*-aminophenol were recovered. The filtrate from the second crop of crystals was now evaporated to a paste and the solid matter separated and benzoylated. It yielded 20 mg. of *ON*-dibenzoyl-*p*-aminophenol (m.p. 229–230°). The mother liquor of the above paste was now stirred with 2N-NaOH and then extracted with ether. The ether yielded nothing. The alkaline liquor was made less alkaline by acidifying and then adding Na_2CO_3. Extraction with ether yielded a small residue, which on benzoylation yielded 1 mg. of dibenzoyl-*p*-aminophenol (m.p. 225–230°). No trace of *o*-aminophenol was found (*ON*-dibenzoyl-*o*-aminophenol has m.p. 180°). Further extraction of the urine with ether for 8 hr. yielded a small amount of *p*-aminophenol.

The total amount of *p*-aminophenol obtained by this mild hydrolysis was equivalent to 11% of the acetanilide fed. Quantitative determinations of the ethereal-sulphate output showed that 12% of acetanilide was excreted as a sulphate (see Table 1). It is therefore concluded that the ethereal-sulphate fraction of acetanilide urine contains no other phenol than *p*-aminophenol. Since very little of free amino compounds is excreted in acetanilide urine the actual phenol excreted as a sulphate is *p*-acetamidophenol (cf. Mörner, 1889).

(*e*) *The glucuronide fraction.* (1) *Detection of p-aminophenol.* Two rabbits were each fed 2 g. of acetanilide and the urine collected for 24 hr. The urine was made faintly acid with acetic acid and treated with saturated normal lead acetate. The precipitate was removed and discarded. The filtrate was made faintly alkaline and basic lead acetate added until precipitation was complete. The basic lead precipitate was filtered and washed with water. It was then suspended in water and the lead removed with H_2S. After removal of the PbS, the filtrate was evaporated with addition of ethanol *in vacuo* at 40–50° to a thin syrup. A small amount of inorganic material was removed from this syrup by diluting with ethanol and filtering. On addition of ether or chloroform to the ethanolic solution, the glucuronide was thrown down as amorphous hygroscopic flocks. In four separate experiments this material could not be induced to crystallize. The glucuronide gum gave only a very feeble diazo reaction indicating the almost total absence of free aromatic amino groups. On keeping a small portion of the gum with conc. HCl at room temperature there was no change in the diazo reaction.

The gum obtained after the feeding of 4 g. of acetanilide was now boiled for 1 hr. with 100 ml. 5N-HCl. The resulting solution gave an intense *p*-aminophenol type of diazo reaction (i.e. on diazotizing and coupling with dimethyl-α-naphthylamine there is obtained at first a fleeting dirty blue colour and then, on standing, the solution becomes a deep blue colour). The solution was made just alkaline and extracted with ether in a separatory funnel. The ether extract was evaporated and the residue benzoylated. There were obtained 800 mg. of *ON*-dibenzoyl-*p*-aminophenol (m.p. and mixed m.p. 234°) after recrystallization from ethanol. The yield was 5% of the dose of acetanilide.

(2) *Acetylation and methylation of the glucuronide gum.* The gum from the urine of rabbits after the feeding of 10 g. of acetanilide was divided into two equal parts. The first part was methylated with methyl sulphate and alkali followed by methyl iodide and Ag_2O. The product was an intractable chloroform-soluble gum which was not further studied.

The second part was dissolved in 100 ml. of a 50 : 50 pyridine-acetic anhydride mixture and left overnight at room temperature. The mixture was then poured into 1 l. of dilute HCl, and the solution extracted with chloroform.

The chloroform extract was dried with anhyd. $CaCl_2$ and evaporated on a water bath. From the residue there were obtained 600 mg. (3·5% of the dose) of a white amorphous solid. This solid recrystallized from acetone water as needles, m.p. 224° (decomp.). It was acid to litmus and gave the naphthoresorcinol reaction on prolonged boiling. The diazo reaction was negative, but was positive after hydrolysis. It was laevorotatory, $[\alpha]_D^{20^\circ} = -15\cdot1^\circ$ (*c*, 2·8 in acetone). This compound appears to be *p-acetamidophenyltriacetylglucuronic acid*. (Found: C, 52·7; H, 5·2; N, 3·7%. $C_{20}H_{23}O_{11}N$ requires C, 53·0; H, 5·1; N, 3·1%.) (Ultraviolet absorption spectrum: $\lambda_{max.}$ 245 mμ.; $\epsilon_{max.}$ 18,000 in ethanol (see Fig. 1).)

300 mg. of the above acid dissolved in acetone were methylated with methyl iodide and Ag_2O at room temperature overnight. Removal of the solvent yielded an *ester* (210 mg.) which crystallized from ethanol in small sheaves of rods with m.p. 210° and showing $[\alpha]_D^{25^\circ} = -22\cdot5^\circ$ (*c*, 3·3 in chloroform). (Found: C, 54·05; H, 5·55; N, 3·4%. $C_{21}H_{25}O_{11}N$ requires C, 54·0; H, 5·4; N, 3·0%.) Its ultraviolet absorption spectrum in ethanol (see Fig. 1) was determined, and was found to contain a band with $\lambda_{max.}$ at 246 mμ.; $\epsilon_{max.}$ 15,600. It was neutral in reaction, sparingly soluble in water, but easily soluble in acetone and ethanol. It was identified as the *methyl ester* of *p-acetamidophenyltriacetyl-β-D-glucuronic acid*, for it did not depress the melting point of an authentic sample of this ester and was identical with it in all respects (see below).

(3) *Isolation of the benzylamine salt of p-acetamidophenylglucuronide.* Identification of the glucuronide of acetanilide urine was finally established through the use of benzylamine. It was found that under certain conditions aminophenylglucuronides could be isolated from solutions in almost quantitative yields as crystalline benzylamine salts. This observation was made after testing a large number of bases including brucine, *o*-toluidine, benzidine, aniline, benzylthiourea and guanidine.

A rabbit was given 2 g. of acetanilide orally and its urine (225 ml.) was collected during the next 24 hr. This urine was estimated to contain about 1 g. of glucuronic acid more than normal. The glucuronide gum was prepared by systematic lead precipitation as already described. The gum was freed from small amounts of inorganic material by dissolution in ethanol and filtering. The purified gum was now dissolved in sufficient aqueous ethanol to produce a 5% solution of the gum. To this solution was added slightly more than one equivalent of benzylamine. Then ethyl acetate and ethanol were added alternately so that the gum concentration was 0·1–0·5% and enough ethyl acetate had been added to make the solution cloudy. The mixture was then allowed to stand at 0° for a few days. The crystalline precipitate (1·03 g., m.p. 196–197°) was filtered off and washed with ethanol. The filtrate was taken down and then diluted with ethanol-ethyl acetate as before. On standing a further 0·35 g. of crystals (m.p. 195–196°) was obtained. Repetition of the process yielded another 0·21 g. The total material recovered was 1·59 g. or 71% of the extra glucuronic acid in the urine. The crude salt gave a very faint diazo reaction which disappeared after one recrystallization from 95% ethanol. The salt showed $[\alpha]_D^{25^\circ} = -63\cdot8^\circ$ (*c*, 4·7 in water), m.p. 195–197° and mixed m.p. 195–197° with authentic benzylamine *p*-acetamidophenylglucuronate (see below). (Found: C, 55·3; H, 6·2; N, 6·5%. $C_{21}H_{26}O_8N_2 . H_2O$ requires C, 55·7; H, 6·2; N, 6·2%.)

PREPARATION OF REFERENCE COMPOUNDS

Benzylamine salt of p-acetamidophenylglucuronide. Four rabbits were each fed 1·5 g. of *p*-acetamidophenol by stomach tube. This phenol is rapidly conjugated and excreted and therefore the urine was collected for 3–4 hr. after feeding. The basic lead acetate precipitate was obtained in the usual manner and the Pb removed by H_2S. The filtrate from the PbS gave, on concentration, *p*-acetamidophenylglucuronide as a gum which could not be crystallized. (Crystalline *p*-acetamidophenylglucuronide was obtained by acetylation of *p*-aminophenylglucuronide (see below).) The gum was dissolved in 3 vol. of ethanol and benzylamine and a mixture of ethyl acetate and ethanol was added to give a cloudy solution with a concentration of 0·1% as regards the gum. On standing at 0° the *benzylamine salt* crystallized out as needles (yield, 0·5 g.). The salt was recrystallized from water-ethanol-ethyl acetate mixtures, and had m.p. 195–197° and $[\alpha]_D^{25^\circ}, = -64\cdot1^\circ$ (*c*, 4·4 in water). (Found: C 55·9; H, 6·3; N, 6·4%. $C_{21}H_{26}O_8N_2 . H_2O$ requires C, 55·7; H, 6·2; N, 6·2%.) It was very soluble in water but insoluble in dry organic solvents.

Benzylamine salt of p-aminophenylglucuronide. *p*-Aminophenylglucuronide (m.p. 215–216°; $[\alpha]_D = -82\cdot7^\circ$ in dilute HCl) was prepared according to Williams (1943). From the urine collected during the first 3 hr. after feeding 10 g. of *p*-aminophenol, 3·3 g. of the glucuronide were isolated. For the 24 hr. urine the yield of crystalline glucuronide was 3·95 g. or 13% of the dose. To prepare the benzylamine salt, the glucuronide was suspended in a small amount of water and slight excess of benzylamine added. This was followed by 5 vol. ethanol and then ethyl acetate until the solution became cloudy. On standing at 0°, the *benzylamine salt* of *p-aminophenylglucuronide* separated as square plates. This was recrystallized from the same solvents, and had m.p. 211–212° and $[\alpha]_D^{20^\circ} = -62\cdot7^\circ$ (*c*, 6·5 in 0·2 N-HCl). (Found: C, 53·1; H, 6·6; N, 6·4%. $C_{19}H_{24}O_7N_2 . 2H_2O$ requires C, 53·3; H, 6·6; N, 6·5%.)

p-Acetamidophenylglucuronide. 2 g. of *p*-aminophenylglucuronide were dissolved in excess dilute Na_2CO_3 solution and shaken with an excess of acetic anhydride until tests for free aromatic NH_2 groups were negative. The *N*-acetylated glucuronide was obtained from the solution as the insoluble lead salt by addition of basic lead acetate solution. The lead salt, suspended in water, was freed from Pb by H_2S. The filtrate from the PbS was concentrated *in vacuo* to a clear syrupy solution (5–10 ml.). To this were added 100 ml. ethanol and the mixture was concentrated *in vacuo* at 40° to 5 ml. 50 ml. ethanol were now added followed by enough ether to give a slight precipitate. The whole was now kept at 0° for 48 hr. whereby *p-acetamidophenylglucuronide* (0·7 g.) crystallized as long, narrow plates. It was recrystallized from hot 95% ethanol (m.p. 193°, decomp. after sintering at 100°); $[\alpha]_D^{22^\circ} = -63\cdot9^\circ$ (*c*, 7·5 in water). (Found: C, 45·3; H, 6·2; N, 3·95%. $C_{14}H_{17}O_8N . 2\frac{1}{2}H_2O$ requires C, 45·2; H, 6·0; N, 3·8%.) It was very soluble in water, soluble in ethanol and insoluble in ether. It did not convert haemoglobin to methaemoglobin *in vitro* at 37° (cf. Williams, 1943).

p-Acetamidophenyltriacetylglucuronic acid methyl ester. 300 mg. of *p*-acetamidophenylglucuronide were dissolved in 50 ml. of absolute ethanol and an ethereal solution of diazomethane (prepared from 1 g. of nitrosomethylurea) added. After 3 hr. the yellow solution was filtered and evaporated *in vacuo* leaving a white, amorphous, hygroscopic solid which was neutral to litmus. This was dissolved in 5 ml. of pyridine and then 5 ml. of acetic anhydride were added. After standing overnight, the mixture was diluted with 50 ml. of water and cooled to 0° for 2 hr. A mass of silky white needles (190 mg.) was deposited. A further 80 mg. were obtained by extracting the mother liquor with chloroform, and crystallizing from water. The product at this stage was apparently a *hydrate* for it melted at 100°, and then resolidified to a glass which melted indefinitely at 150–200°. Drying at 110° in air led to some decomposition because the material turned brown. The anhydrous product was obtained by dissolving the hydrate in absolute ethanol and scratching the beaker, whereby the *methyl ester* of *p-acetamidophenyltriacetylglucuronide* crystallized as sheaves of short needles, m.p. 200–205° and showing $[\alpha]_D^{25^\circ} = -22{\cdot}1^\circ$ (*c*, 7 in chloroform). (Found: C, 54·5; H, 5·6; N, 2·9%. $C_{21}H_{25}O_{11}N$ requires C, 54·0; H, 5·4; N, 3·0%.) Ultraviolet absorption spectrum, $\lambda_{\max.}$ 246 mμ., with $\epsilon_{\max}$ 15,600. The compound is very soluble in acetone, ethanol and chloroform, but sparingly soluble in water and ether.

Benzylamine salt of o-aminophenylglucuronide. o-Aminophenylglucuronide (decomp. at 300°; $[\alpha]_D = -76{\cdot}1^\circ$ in dilute HCl) was prepared by feeding rabbits with *o*-aminophenol according to Williams (1943). The *benzylamine salt* was prepared as for the *p*-isomer. The salt formed clumps of short needles, m.p. 204–205° and $[\alpha]_D^{20^\circ} = -61{\cdot}3^\circ$ (*c*, 6·1 in water). (Found: C, 58·0; H, 6·1; N, 6·9%. $C_{19}H_{24}O_7N_2$ requires C, 58·2; H, 6·1; N, 7·1%.)

SPECTROSCOPIC OBSERVATIONS

The ultraviolet absorption spectra of *p*-aminophenylglucuronide and its acetyl derivatives and *p*-acetamidophenol are recorded in Fig. 1. From this figure it can be seen that derivatives of *p*-aminophenol can be readily distinguished from those of *p*-acetamidophenol. Whereas the spectra of *p*-aminophenol (see Williams, 1947; Morton & Stubbs, 1940) and *p*-aminophenylglucuronide show two maxima in the region 220–320 mμ., with molecular extinction coefficients less than 10,000, all the derivatives of *p*-acetamidophenol exhibit only one band in this region with an extinction coefficient greater than 10,000. *p*-Acetamidophenol itself shows a band at $\lambda_{\max.}$ 250 mμ., $\epsilon_{\max.}$ 13,800 and an inflexion at 285 mμ., ε 3500, whereas *p*-aminophenol shows two bands at $\lambda_{\max.}$ 234 and 395 mμ. with $\epsilon_{\max.}$ 7950 and 2700 respectively. For the glucuronides, the figures are: *p*-acetamidophenylglucuronide, $\lambda_{\max.}$ 243 mμ., $\epsilon_{\max.}$ 10,800 and *p*-aminophenylglucuronide, $\lambda_{\max.}$ 231 and 291 mμ., $\epsilon_{\max.}$ 8700 and 1600.

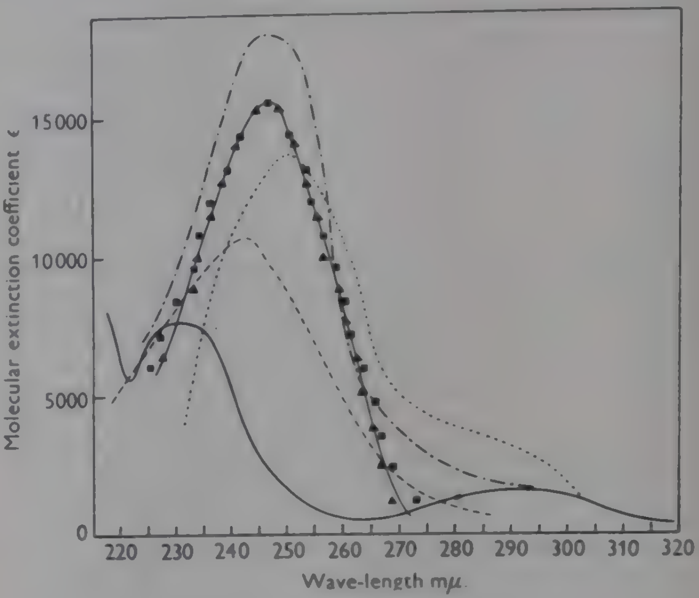
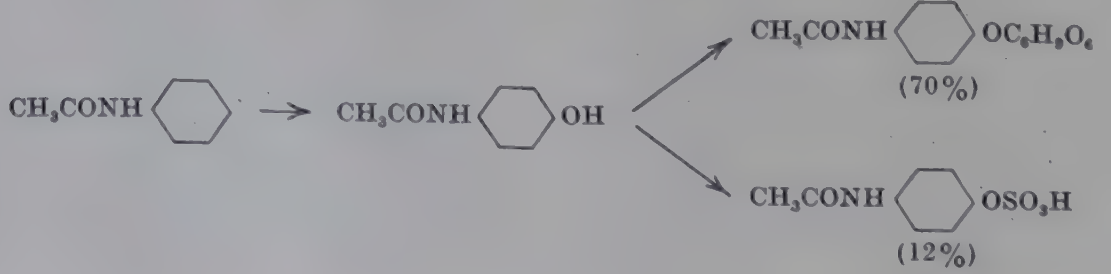


Fig. 1. Ultraviolet absorption spectra of *p*-aminophenylglucuronide and related compounds. ···· *p*-acetamidophenol in ethanol: $\lambda_{\max.}$ 250 mμ., $\epsilon_{\max.}$ 13,800; ~ 285 mμ., ε 3500. - - - - - *p*-acetamidophenylglucuronide in water: $\lambda_{\max.}$ 243 mμ., $\epsilon_{\max.}$ 10,800. –·–·– *p*-acetamidophenyltriacetylglucuronic acid in ethanol: $\lambda_{\max.}$ 245 mμ., $\epsilon_{\max.}$ 18,000. ■—■ *p*-acetamidophenyltriacetylglucuronide methyl ester (synthetic) in ethanol: $\lambda_{\max.}$ 246 mμ., $\epsilon_{\max.}$ 15,600. ▲—▲ *p*-acetamidophenyltriacetylglucuronide methyl ester (from acetanilide urine) in ethanol: $\lambda_{\max.}$ 246 mμ., $\epsilon_{\max.}$ 15,600. —— *p*-aminophenylglucuronide in 0·1 N-KOH: $\lambda_{\max.}$ 231 and 291 mμ., $\epsilon_{\max.}$ 8700 and 1600.

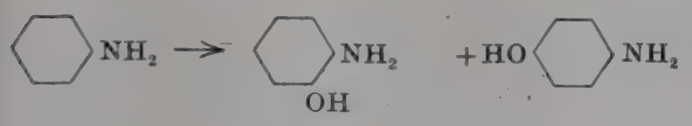
DISCUSSION

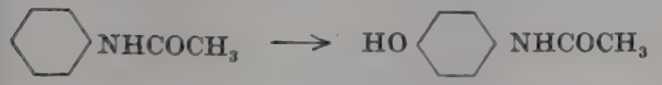
The present work proves that the metabolites of acetanilide in the rabbit are *p*-acetamidophenylglucuronide and *p*-acetamidophenylsulphuric acid which are excreted in the ratio of about 6 to 1 and account for over 80% of the acetanilide fed. Small traces of acetanilide (*c.* 0·2% of dose) and of aniline (*c.* 0·2%) are also found in the urine. The metabolism of acetanilide in the rabbit can therefore be represented as follows:



Acetanilide undergoes very little deacetylation (less than 5 % of the dose as determined by diazotization and coupling), an observation which agrees with our earlier results on *p*-substituted acetanilides (Smith & Williams, 1948). Michel *et al.* (1937) have shown that acetanilide is deacetylated by isolated rat tissues. Our results indicate that in the intact rabbit either such a deacetylating system does not occur or, if it does, it has no opportunity of functioning to any great extent. Some animals may, however, be able to deacetylate the aromatic acetamido group, for Sykes (1944) has shown that whereas N^4-acetylsulphamezathine is excreted almost entirely unchanged by man, 40 % is deacetylated in the pigeon. Whether or not an acetamido compound is deacetylated *in vivo* may have an important bearing on its fate in the body, as will be seen from what follows.

We have found (Smith & Williams, unpublished) that in rabbits aniline is oxidized but is not acetylated, and the products of oxidation are *o*- and *p*-aminophenols (together with small amounts of what we think is 2:4-dihydroxyaniline). No trace of *o*-aminophenol or its *N*-acetyl derivative was detected in acetanilide urine:





Present views (see Pauling, 1945) concerning the effect of substituents on the activity of aromatic rings in substitution reactions indicate that the amino group in aniline activates, mainly through resonance, the ortho and para positions. It is to be expected therefore that biological oxidation will take place in these positions. This actually occurs with aniline in the rabbit. In the case of acetanilide, biological oxidation occurs only in the para position. This result is similar to the behaviour of acetanilide on nitration which gives rise to the formation of 95 % *p*- and 5 % *o*-nitroacetanilide, whereas nitration of aniline gives 50 % *p*- and 40 % *o*-nitro compound (Cumming, Hopper & Wheeler, 1937). Now acetylation of the amino group of aniline will result in some deactivation of the ring. This deactivation is more pronounced in the *o*-position than in the *p*-position through the so-called inductive effect of the acetamido group working in opposition to the resonance effect. In addition to this, the acetamido group is larger than the amino group and will therefore exert a steric hindrance effect on ortho substitution. From these arguments it can be seen that ortho substitution of acetanilide may be hindered both by steric hindrance and by deactivation through induction. For biological oxidation to take place, it may be assumed that a minimum activation of the position to be oxidized is necessary. The absence of biological oxidation of acetanilide in the ortho position could therefore be explained on the grounds that (1) the ortho positions are not sufficiently activated for biological oxidation, and (2) steric hindrance due to the acetamino group prevents the approach of the biological oxidizing system to the ortho positions. Oxidation of acetanilide should therefore take place in the para position, for here there is no steric hindrance and the position is sufficiently activated, through resonance, for the enzyme system to attack it.

It follows from the above arguments that if an animal can deacetylate acetanilide to aniline, then it is to be expected that acetanilide will give rise to *o*- and *p*-aminophenol in such an animal. When acetanilide is fed to dogs, both *o*- and *p*-aminophenol can be detected in the urine (Jaffé & Hilbert, 1888). It is probable that the dog possesses an active deacetylating enzyme for aromatic acetamido compounds.

The fate of acetanilide in man (cf. Mörner, 1889) is probably very similar to that in the rabbit. Lester & Greenberg (1947) have recently shown that the major metabolites of acetanilide in man are *p*-acetamidophenol and its *O* conjugates, although they did not isolate these compounds.

SUMMARY

1. It has been shown that, in the rabbit, acetanilide is oxidized almost entirely to *p*-acetamidophenol, which is excreted in the urine as *p*-acetamidophenylglucuronide and *p*-acetamidophenylsulphuric acid, these occurring in the ratio 6 : 1.

2. Less than 5 % of the acetanilide fed is deacetylated and only traces of aniline and unchanged acetanilide were isolated.

3. *p*-Acetamidophenylglucuronide can be conveniently isolated as the benzylamine salt.

4. The following reference compounds have been synthesized: the benzylamine salts of *o*- and *p*-aminophenylglucuronides and of *p*-acetamidophenylglucuronide; *p*-acetamidophenylglucuronide and the triacetyl derivative of its methyl ester.

5. The ultraviolet absorption spectra of the compounds studied have been recorded and discussed.

6. The theoretical implications of the biological oxidation of acetanilide have also been discussed.

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The Molecular Weight of Insulin and its Dependence upon pH, Concentration and Temperature

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In a previous paper (Gutfreund, 1948) it was shown from the results of osmotic and diffusion-sedimentation measurements that the molecular weight of insulin is 47,000–48,000 in solutions of pH 7·0 containing 0·5–1·0% protein. Molecules of that size were found to dissociate into smaller units on dilution to protein concentrations of 0·2% or less. Sjögren & Svedberg (1931) have determined the sedimentation constant of insulin over a range of pH from 3·5 to 12·3 and found from their data that the molecular weight has a maximum at pH 6·8. They concluded that insulin dissociates both in acid and in alkaline solutions and also that this dissociation is reversible if the solution is not kept too long at a pH outside the stability region. Gutfreund & Ogston (1946) re-examined the sedimentation constant over a range of pH from 3·5 to 9·6 and found that the sedimentation velocity of the main boundary remained constant between pH 6·7 and 9·6. In some cases, however, some slower-sedimenting material was present.

Gutfreund & Ogston were interested in the sedimentation rate of the main boundary. A re-examination of the sedimentation diagrams showed the presence, in solutions more alkaline than pH 8, of some more slowly sedimenting material which did not form a clear boundary.

The recent work of Waugh (1944, 1945, 1946) showed that insulin undergoes drastic and reversible changes in acid solutions, and the studies of the influence of pH on the properties of insulin solutions have therefore been continued and extended by means of both the osmotic pressure and ultra centrifugal technique.

EXPERIMENTAL

The sedimentation constants were determined at Oxford in the oil-turbine ultracentrifuge (Svedberg type). The sedimenting boundaries were observed by the Philpot (1938) method. The speed of the centrifuge during all the experiments was about 1000 rev./sec. A detailed description of the accuracy of the measurements is given by Gutfreund & Ogston (1946). All values of sedimentation constants (S_{20}) recorded are corrected to sedimentation in water at 20°.

For the osmotic-pressure measurements an apparatus of the type described by Adair (1925) was used. The pressure was measured directly as the height of solution in the capillary tube. The protein concentration of the equilibrated solution was calculated from Δn (refractive index difference between buffer and solution) assuming a specific refractive increment of 180×10^{-5}. Δn was measured in a Zeiss interferometer, the usual type of refractometers not being accurate enough for measurements on the very dilute solutions used throughout this work.

Since the solutions were very dilute all the values for molecular weights, recorded in this paper, were calculated by means of the simple formula

$$M=\frac{RTC}{P},$$

where M = molecular weight; P = osmotic pressure; R = gas constant; C = concentration of protein in g./l. of solution; and T = absolute temperature. Except where explicitly stated, the osmotic pressure experiments were carried out in a cold room at +2°.

The insulin used for all the investigations described in this paper was a sample of crystalline zinc insulin (Boots Pure Drug Co. Ltd.). Ultracentrifugal examination (Gutfreund & Ogston, 1946) showed that this material was homogeneous in solutions of pH 7·0–8·0. Electrophoretic analysis in the Tiselius apparatus, using the Philpot (1938) optical system for the observation of the moving boundaries, also confirmed the purity of the sample used.

Table 1. *Sedimentation constants of insulin*

Exp. no.	Insulin concentration (%)	pH	Molar composition of buffer solution: Citric acid	HCl	NaCl	Na_2HPO_4	S_{20} (corr.) × 10^{13}
1*	0·4	3·4	0·055	—	0·1	0·045	3·10
2	0·6	3·0	0·079	—	0·2	0·041	3·36
3	0·15	2·5	0·091	—	0·2	0·017	—
4	0·75	2·2	0·098	—	0·2	0·004	2·59
5	1·20	1·0	—	0·1	0·2	—	2·78
6†	0·84	8·0	—	—	0·1	0·0095 + 0·0005 KH_2PO_4	3·47

* Quoted from Gutfreund & Ogston (1946).

† Solution used in Exp. 5 adjusted to pH 8 and dialyzed against phosphate buffer (see text).

Two types of buffer systems were employed as solvent: for solutions of pH 2·2–5·0, McIlvaine's (1921) citric acid-phosphate buffer; and for those of pH 5·0–8·8, mixtures of Na_2HPO_4 and KH_2PO_4 as described by Clark (1928). Solutions of pH 1 were prepared in 0·1 M-HCl.

After the protein solutions had been dialyzed against the appropriate buffer, the pH of the dialysate was determined with a glass electrode.

RESULTS

Gutfreund & Ogston (1946) found that insulin was polydisperse in solution of pH 3·4 and the first series of experiments described here was carried out to extend the study on the properties of more acidic insulin solutions. In Table 1 the sedimentation constants calculated from measurements on solutions of pH 3·4, 3·0, 2·2 and 1·0 respectively, are recorded together with details about composition of the buffer used as solvent and the protein concentration. Specimen photographs of the boundaries are shown in Fig. 1.

It can be seen from the width of the sedimenting boundaries shown in the sedimentation diagrams 1, 2, 3 and 4 that insulin in acid solutions is heterogeneous with respect to particle size. It can be presumed that these solutions contain insulin in various states of aggregation or dissociation. The spread of the heterogeneous boundaries decreases the accuracy of the values for S_{20}, particularly for the more dilute solutions. This, as well as dissociation due to dilution, may account for the difference in the value for S_{20} obtained from the ultracentrifuge Exps. 1 and 2. From Exp. 4 a lower sedimentation constant was calculated for the main boundary and the presence of a considerable amount of very slowly sedimenting material is evident from the boundary diagram. In a solution of pH 1·0 (Exp. 5) the sedimentation constant was somewhat higher than in a solution of pH 2·2. Furthermore, the boundary in the sedimentation diagram for Exp. 5 has a much more homogeneous appearance than that derived from all the other experiments on insulin in acid solutions.

The combined effect of dilution and acidity on insulin was also investigated. A solution containing 0·15 % insulin in buffer of pH 2·5 was examined in the ultracentrifuge (Exp. 3) and the sedimentation diagram shows clearly a marked increase in the proportion of very slowly sedimenting material compared with the more concentrated solution of Exp. 4.

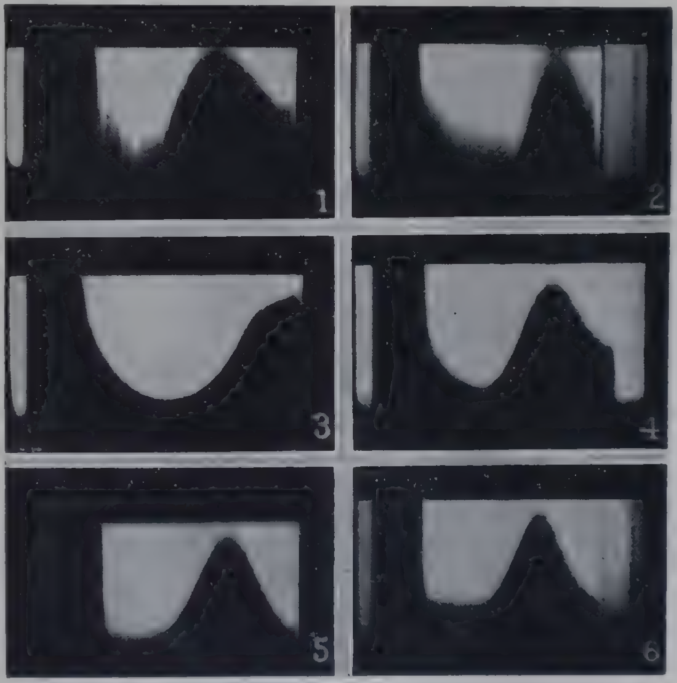

Fig. 1. Sedimentation diagrams for Exps. 1–6 numbered as in Table 1. The time (in min.) after reaching full speed was 80, 57, 80, 80, 60, 60, for diagrams 1–6 respectively.

As no clear boundary was formed the sedimentation constant could not be computed from Exp. 3.

The solution of pH 1·0, which had been used for sedimentation Exp. 5, was afterwards adjusted to pH 8·0, and dialyzed against phosphate buffer of that pH and again examined in the ultracentrifuge (Exp. 6); the sedimentation constant of insulin in this solution, $S_{20} \times 10^{13}$, was 3·47, which agrees within the experimental error with the value recorded for the sedimentation constant of unchanged insulin.

In the previous paper on the osmotic pressure of insulin solutions (Gutfreund, 1948) experiments were described in which phosphate buffer of pH 7·0 was used as solvent. It was necessary to dilute the buffer to 0·01 M to increase the solubility of insulin. In such dilute buffer a quite appreciable partial pressure due to excess ion activity was observed, and the necessary corrections were calculated from the results of membrane-potential measurements. In this paper the extrapolation procedure suggested by Adair & Robinson (1930) is used to interpret the results from measurements on solutions of insulin in 0·01 M-phosphate buffer of pH 7·5. The graph in Fig. 2 shows P/C plotted against C and gives several interesting results. At the centre of the curve (C equal to 0·4–0·5 %) $P/C = 4{\cdot}9$, corresponding to a molecular weight of 47,400, which is in good agreement with the value previously obtained at pH 7·0 and similar protein concentration (0·4–0·8 %). The value for P/C increases however both for $C > 0{\cdot}5$ and $C < 0{\cdot}3$. This is due to the membrane-potential effect in the more concentrated solutions and to the effect of dissociation, which has previously been described (Gutfreund, 1948) in the very dilute solutions.

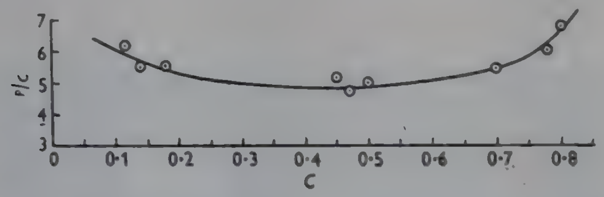

Fig. 2. Plot of P/C against C from osmotic-pressure measurements on solutions of insulin in 0·01 M-phosphate buffer of pH 7·5. P = osmotic pressure; C = concentration of protein in g./100 ml. solution.

Between pH 4·0–7·0 insulin is so insoluble as to make any investigations on solutions very difficult. Between pH 7·0 and 7·5 solutions up to nearly 1 % could be obtained in very dilute buffer. At pH lower than 4·0 and higher than 7·5 it was possible to obtain insulin solutions, containing up to 1 % protein, in the presence of sufficient salt to eliminate the partial pressure due to excess ion activity. I have therefore made all the buffer solutions of those outside pH regions, 0·2 M with respect to NaCl. The following series of experiments was carried out at pH 1 (0·1 M-HCl, 0·2 M-NaCl) to find out whether P/C remained constant over a range of concentrations 0·5–1·0 % of protein, under those conditions.

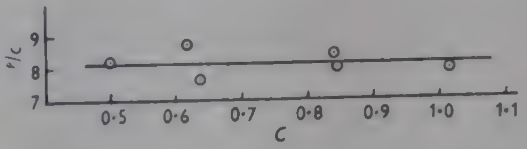

Fig. 3. Plot of P/C against C from osmotic-pressure measurements on solutions of insulin in 0·1 M-HCl, 0·2 M-NaCl. P = osmotic pressure; C = concentration of protein in g./100 ml. solution.

Fig. 3 is a graphical representation of the measurements on solutions of pH 1. The molecular weight, calculated from the results of the six experiments recorded here, is 28,500 ± 1100 (standard deviation from the mean). The ratio P/C is constant over the range of C investigated, and it can therefore be concluded that the low molecular weight observed is due to dissociation in acid solutions and not to the effect of the electric charge of the protein molecules. These experiments also demonstrate the accuracy to be expected from osmotic-pressure measurements on solutions of similar concentration.

A series of measurements of the osmotic pressure of insulin solutions (protein concentration 0·6–0·9 %) over a range of pH from 1·0 to 8·8 was then carried out, and the molecular weight values, recorded in Table 2, are in each case the means of the results calculated from several osmotic-pressure measurements.

Table 2. *The dependence of the molecular weight of insulin, in solutions of protein concentration* 0·6–0·9 %, *upon the* pH *of the solution*

pH	Molecular weight calculated from osmotic-pressure measurements
1·0	28,500
2·2	22,000
2·5	22,000
3·0	22,000
7·0	47,700
7·5	47,400
8·0	40,000
8·8	28,000

It is clearly indicated by these observations that insulin dissociates into subunits, both in acid and alkaline solutions. The reversibility of this dissociation was examined by adjusting both acid and alkaline solutions of insulin to pH 7·5 and it was found that re-association to a molecular weight of 47,000–48,000 occurred.

The observed re-aggregation of the insulin subunits between pH 2·2 and 1·0 suggested that it might be possible to study the fibrous polymerization in acid solutions described by Waugh (1946). However, on changing the concentration of HCl from 0·1 to 1·0 M most of the insulin in solution was precipitated and no measurements were made on more acidic solutions.

An observation, indicating the presence simultaneously of insulin in different states of aggregation, was then made. A buffer solution of pH 8·0 was saturated with insulin at a temperature of 30°. This solution, containing after centrifugation 1·1 % insulin, was stored in a cold room over night and a crop of crystals was formed. Though the mean molecular weight of insulin in this solution was 40,000, the crystals which deposited were found to be isomorphous with those described by Crowfoot (1938).

From the results of the experiments so far described it is clear that in acid solutions the pH of maximum dissociation is between 2 and 3. The results of the ultracentrifuge Exps. 3 and 4 show that the degree of dissociation is dependent upon insulin concentration. The combined effect of pH and dilution was studied by a series of osmotic-pressure measurements on solutions of pH 2·5 containing 0·04–2·4 % of insulin. The results of these experiments are recorded in Fig. 4 where the molecular weight is plotted against insulin concentration. Two curves are shown: one represents the results of measurements at 2°, the other those of measurements at 23°. Both curves can be extrapolated to a value of 12,000 ± 500 (standard deviation of the individual observations) for the molecular weight at zero concentration. A consideration of the accuracy of the measurements is particularly important for very dilute solutions (0·04–0·1 % of protein). The accuracy of the molecular weight calculated from an osmotic-pressure measurement depends upon the accuracy of the estimation of the protein concentration and pressure. For the smallest values of concentration and pressure, measured for the calculation of the data in Fig. 4, the accuracy was ± 5 % in each case.

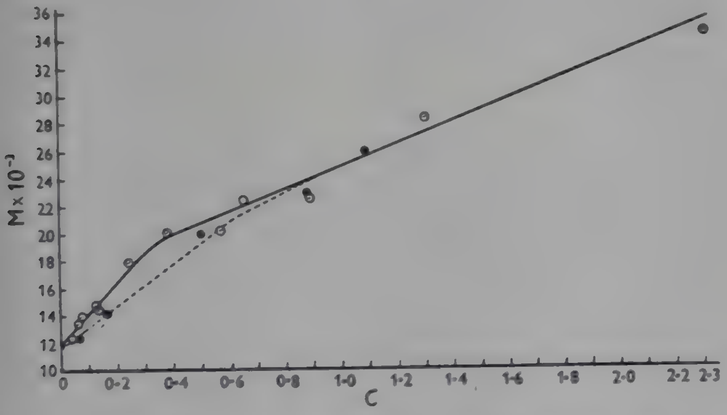

Fig. 4. Plot of molecular weight of insulin against concentration calculated from osmotic-pressure measurements in citric acid-phosphate buffer (pH 2·5). Empty circles represent measurements at 2°, full circles represent measurements at 23°.

DISCUSSION

From the results reported in this paper and from the data given by Gutfreund (1948) it can be concluded that the maximum molecular weight of insulin is 48,000. This value is found for neutral solutions (pH 7·0–7·5) of protein concentration 0·4–0·9 %. Under these special conditions insulin is homogeneous with respect to particle size. In the previous paper it has been shown that insulin of molecular weight 48,000 is not very stable, as dissociation into smaller units occurs on dilution to protein concentrations of less than 0·3 %.

The investigation of the effect of pH on the molecular weight of insulin showed that dissociation of insulin occurred both in acid and alkaline solutions. In acid solutions the optimum pH for dissociation is pH 2·0–3·0. pH 1 is the critical pH for the beginning of the re-aggregation resulting in more acidic solutions, in the formation of a fibrous precipitate. The results of ultracentrifugal and osmotic-pressure measurements are in agreement about the dissociation in acid solutions and the beginning of re-aggregation in solutions of pH 1. When establishment of equilibrium between dissociated and associated insulin molecules is not slower than the resolution produced by sedimentation, one can expect to get a spreading boundary giving a mean sedimentation constant, rather than two or more separate component boundaries. It is at present difficult to give a definite explanation for the results obtained by Gutfreund & Ogston (1946) on ultracentrifugal examination of insulin in alkaline solutions. They observed no change in the sedimentation constant nor any spreading of the boundary. However, as stated on p. 544 of this paper, some slowly-sedimenting material could be seen in the sedimentation diagrams from experiments on solutions more alkaline than pH 8·0. It is possible that the difference in the appearance of sedimenting boundaries in acid and alkaline solutions is due to a difference in the speed of the dissociation equilibrium under the two respective conditions. A very slow equilibrium may account for the type of boundaries obtained in alkaline solutions.

The last series of osmotic-pressure measurements described in this paper was undertaken to investigate the size of the units into which insulin dissociates. Though the results indicate that the molecular weight of insulin in infinitely dilute solution is 12,000, there is no direct proof from these data as to the homogeneity of the dissociation products. In spite of variations of temperature, pH and concentration, the molecular weight was never found to be below 12,000 and the two curves from measurements at 2° and 23° converge at the point of molecular weight 12,000 (Fig. 4); this can be assumed to be the molecular weight of the dissociation product.

The results of the crystallographic investigations (Crowfoot, 1938) and of the chemical analysis (Chibnall, 1946) do not by themselves prove the existence of a subunit of molecular weight 12,000, but they are consistent with that hypothesis. The unit cell in crystalline insulin is composed of three identical parts and the unit-cell weight is 36,000 (recalculated for a moisture content of 10–11 % for insulin crystals). The minimum molecular weight calculated from amino-acid analysis is 12,100 with a standard deviation of 530, from the values quoted by Chibnall (1946).

The reversibility of the dissociation reaction of insulin was demonstrated by the ultracentrifugal Exps. 5 and 6, by osmotic-pressure measurements and by the work of Waugh (1946). Gutfreund (1948) calculated the total heat change occurring on dissociation of insulin molecules of molecular weight 48,000 into four identical subunits. At pH 7·0, ΔH was found to be 29,000 g. calories (for 1 mol. insulin). Similar calculations were not done for the data recorded in Fig. 4, as a series of measurements at each temperature for one point of the concentration scale has to be made to get results of sufficient accuracy for subsequent calculation of the relative concentrations of associated and dissociated insulin. The data so far obtained are, however, quite accurate enough to show the relation between concentration and molecular weight.

Since the degree of dissociation of insulin molecules is dependent upon pH, temperature and concentration, it can be concluded that the forces holding the subunits together involve the ionizing groups of the protein. It is very difficult to differentiate between electrostatic forces and hydrogen bonds as the latter are often affected by the ionization of certain groups. A study of the influence of the ionic strength of the solvent buffer upon dissociation, which is now being pursued, may give further information on that point.

SUMMARY

1. Insulin has a maximum molecular weight (47,000–48,000) in solutions of pH 7·0–7·5 at protein concentrations of 0·4–0·9 %. Under those conditions it is homogeneous with respect to particle size.

2. Molecules of this weight dissociate reversibly on change of pH to above 7·5 or below 4, as well as on dilution. The optimum pH for dissociation was found to be 2·0–3·0.

3. On studying the combined effect of pH, dilution and temperature upon dissociation it was found that the minimum molecular weight was 12,000 ± 500. Other evidence suggesting that insulin molecules in solution were composed of subunits of molecular weight 12,000 is summarized.

4. It is suggested that units of molecular weight 12,000 are bound by weak electrostatic forces.

5. The dissociation phenomena have been studied by osmotic pressure and by ultracentrifugal measurements. The two methods gave results in good agreement.

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The Distribution of Acid-soluble Phosphates in the Fatty Liver

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Although increased attention has recently been paid to the function of the acid-soluble phosphates in liver metabolism, the bulk of the investigations has been concerned with changes in the distribution of organic phosphates brought about by variations in diet (Flock, Bollman & Mann, 1936*a*; Kaplan & Greenberg, 1944*b*; Rapoport, Leva & Guest, 1943 *a*, *b*). The effect of injections of glucose (Kaplan & Greenberg, 1944*a*), insulin (Cori & Goltz, 1925; Nelson, Rapoport, Guest & Mirsky, 1942; Kaplan & Greenberg, 1943, 1944*a*) and various enzyme inhibitors has also been studied. Little work, however, appears to have been done on the acid-soluble phosphates of fatty livers, although Flock *et al.* (1936*a*), working with dogs maintained on a high fat diet and thus having fatty livers, have stated that if the diet is given for 3 weeks or longer there is a decrease of inorganic phosphate to 60% of the normal value. The same authors (Flock *et al.* 1936*b*) also subjected dogs to carbon tetrachloride anaesthesia for 1 hr. on four successive days, but found that the distribution of the various acid-soluble-phosphate fractions from the livers was unaffected. The distribution of acid-soluble phosphates from fatty livers has recently become of added interest in view of the importance of adenylic acid in the oxidation of butyrate (Munoz & Leloir, 1943).

It has already been shown that the oxygen consumption and the acetoacetic acid production of liver slices from guinea pigs treated with carbon tetrachloride is considerably in excess of normal (Ennor, 1942). This together with the fact that adenosine triphosphate (*ATP*) is essential for the oxidation of fatty acids (Lehninger, 1945 *a*, *b*) suggested the likelihood of a correlation between the adenosine polyphosphate (*APP*) content and the increased metabolism of these fatty livers. Experiments designed to test this possibility have been carried out and are described in this communication. Preliminary results were reported earlier (Ennor & Stocken, 1947).

EXPERIMENTAL

Expression of results

All the above workers have expressed their results on the basis of wet weight of tissue, but, since it has been shown that the liver is subject to wide variations in water, fat and glycogen content (cf. Higgins, Berkson & Flock, 1932, 1933), it is clear that reference to a weight which includes these substances is open to criticism. Flock *et al.* (1936*a*) commented on this, but were unable to show that calculation on the basis of dry, fat-free weight altered their conclusions. Since variations in the acid-soluble organic phosphates of liver reflect variations in the metabolism of carbohydrate, protein and fat, and thus in the activities of the various enzymes concerned, it seems desirable to express all results in a more satisfactory manner. Expression on the basis of percentage N or nucleoprotein N would largely overcome these objections, and the figures so obtained could be more readily capable of interpretation from the standpoint of metabolic activity. Nucleoprotein N would appear to be the choice, but, because of the technical difficulties associated with its estimation when a large number of other determinations have to be done, all the results quoted in this communication have been expressed on the basis of total liver N. However, for the purpose of comparison with the results of other workers, some of our results are also quoted on the basis of wet liver weight. It is realized that the presence of labile protein in the liver will perhaps complicate the picture, but it is thought that the error introduced will be small, particularly as the mobile protein stores are largely depleted following a 24 hr. fast.

Methods

Phosphorus estimation. Inorganic phosphate was determined by the method of Berenblum & Chain (1938) and total P by the same method after ashing with HNO_3 and H_2SO_4.

Nitrogen. Duplicate samples (*c.* 0·1 g.) of unfrozen liver were ashed with the digestion mixture described by Campbell & Hanna (1937), and after suitable dilution samples were distilled in the apparatus of Markham (1942). The distillate was collected in a boric acid buffer used by Conway (1942) and titrated with 0·01 $\text{N-}H_2SO_4$.

Fat. In this paper 'fat' refers to the material extractable from the liver by means of a 70/30 (v/v) ethanol-diethyl ether mixture.

Creatine. The method used was that of Eggleton, Elsden & Gough (1943).

Pentose. This was estimated according to the method of Meijbaum (1939), but as suggested by Stone (1943) a solution of barium adenosine triphosphate was used as a reference standard.

Treatment of animals. Male guinea pigs, of *c.* 300 g. body weight, fed on a liberal diet of bran, cabbage and swede, were used throughout. Treated animals were given, on successive days, three subcutaneous injections each of 0·2–0·3 ml. of a mixture containing equal volumes of carbon tetrachloride and arachis oil. Free access was allowed to water, but food was withheld from both treated and control animals for 24 hr. prior to the experiment. Treated animals were killed 24 or 48 hr. after the final injection.

Preparation of extracts. The animals were stunned by a blow on the head and the neck vessels severed. The liver was rapidly removed and the major portion dropped into liquid air; the remainder was analyzed for N, total P and fat. After a preliminary grinding in a mortar chilled with liquid air, duplicate samples of 2–3 g. of the frozen liver were homogenized with 4 ml. of ice-cold 10% (w/v) trichloroacetic acid (*TCA*). The homogenizer was similar to that described by Potter & Elvehjem (1936) but was provided with a stainless-steel pestle. The homogenate was diluted with 20 ml. of 5% (w/v) *TCA* and, after centrifugation, the residue was re-extracted with a further 20 ml. of the 5% acid. Since this procedure served to extract not less than 97% of the acid-soluble phosphates, further extraction was considered unnecessary. The combined extracts were made up to a total volume of 50 ml. with 5% *TCA*, filtered if necessary, and analyzed for inorganic and total P.

RESULTS

There is a marked difference in the total-P contents of the fresh livers from the treated and the control animals (Table 1). A similar difference is also apparent when the total- and organic-P contents of the *TCA* extracts are considered. Thus, when the figures are expressed on the basis of the wet weight of the liver, the values for the total P and total and organic acid-soluble P are higher in the control group than in the treated. Expression on the basis of the N content of the liver, however, leads to a reversal of this result.

Table 1. *Nitrogen, phosphorus and fat analyses of livers and liver extracts from control guinea pigs and from guinea pigs treated with carbon tetrachloride*

Fresh liver				*TCA* extract			
		Total P		Total P		Organic P	
Fat (%)	N (%)	(mg./g. N)	(%)	(mg./g. N)	(mg./100 g.)	(mg./g. N)	(mg./100 g.)
			Control animals				
—	3·51	94	0·33	24·7	86·6	17·9	62·6
6·6	3·58	91	0·33	26·5	95·0	20·6	73·9
6·6	3·73	92	0·34	27·4	102·0	21·7	80·7
—	3·54	98	0·35	29·2	102·0	22·8	79·4
7·8	3·63	95	0·34	27·4	99·4	20·6	74·8
6·0	3·06	—	—	27·6	84·6	20·4	62·4
—	3·78	93	0·35	28·3	107·0	22·5	85·4
5·4	3·65	—	—	25·5	93·0	19·1	69·6
9·0	3·57	—	—	27·0	96·5	20·3	72·6
Mean	**3·56**	**94**	**0·34**	**27·1**	**96·2**	**20·7**	**73·5**
			Animals treated with carbon tetrachloride				
16·2	1·84	122	0·23	36·8	67·8	25·4	47·0
12·5	2·20	134	0·29	30·4	66·9	21·9	48·2
10·6	2·46	100	0·24	33·0	82·0	24·4	60·5
13·0	2·30	107	0·24	36·1	83·0	29·0	66·7
14·6	2·20	—	—	35·1	77·3	27·4	60·4
14·5	1·95	—	—	39·8	77·6	31·9	62·2
Mean	**2·16**	**115**	**0·25**	**35·2**	**75·6**	**26·7**	**57·5**

Because of the facts that *ATP* is essential for the oxidation of fat, that there is a well recognized association between oxidation and phosphorylation, and that there is an increase in the rate of O_2 uptake in fatty livers (cf. Meier & Thoenes, 1933; Ennor, 1942), it might be anticipated that there would be an increase in the high energy phosphate levels in such livers. Attention was, therefore, in the first instance, focused on the compounds hydrolyzable in 7 min. in N-acid at 100°. A separation of *APP* from other easily hydrolyzable compounds, such as acetyl phosphate and phosphocreatine, and from many organic phosphates, is possible by barium precipitation and this was carried out on the *TCA* extracts. Several methods have been described for the preliminary separation, but that described by Le Page & Umbreit (1945) is the most convenient. This procedure has the advantage over the alternative one, in which solid baryta is used, in that it is much quicker, but it should be noted that the use of NaOH for the neutralization of *TCA* extracts results in the incomplete precipitation of inorganic phosphate at pH 8·2. Kosterlitz & Ritchie (1943) have commented on this, although it is not clear what concentration of Ba^{++} was present in their extracts. Under our conditions adjustment to pH 9·5, as recommended by these workers, results in a decrease of the solubility of barium phosphate to less than 1 µg. of P/ml. as compared with approximately 2·5 µg. of P/ml. at pH 8·2. In the bulk of our experiments, however, this latter pH has been chosen since the faint pink end point of phenolphthalein is easily reproducible. Further, the small amounts of inorganic phosphate which appear in the mother liquor do not introduce any complication.

Stone (1943) has found that the amount of *ATP* which is precipitable from *TCA* extracts of brain tissue depends on the concentration of $Ca(OH)_2$. Experiments to determine the amount of *ATP* precipitable by barium under our conditions indicated that there was no significant difference in the amounts precipitated when the concentration of Ba^{++} ranged from M/8 to M/30 and that the amount of *ATP* which remained in the mother liquor was less than 0·2 μg. P/ml. The final procedure adopted for the separation was, therefore, as follows.

To 30 ml. of the *TCA* extract was added 1 ml. of 20% (w/v) $BaCl_2$ and sufficient 5 N-NaOH to bring the pH to 8·2 with phenolphthalein as internal indicator. The solution was allowed to stand for 20 min. to ensure complete flocculation and then centrifuged. The supernatant fluid was made up to 50 ml.: the precipitate was dissolved in the minimum volume of 0·1 N-HCl and made up to 25 ml. with water. All manipulations were carried out at 0°. Except where stated the barium was not removed from the various fractions prepared from the *TCA* extracts, since $BaSO_4$ does not interfere with the method used for the estimation of P.

It is clear that there is a marked increase in the 7 min.-hydrolyzable compounds present in the barium-insoluble fraction of *TCA* extracts of the fatty liver. Moreover, this increase is demonstrable whether expressed on the basis of wet weight or N, and in general may be correlated with the fat content (Table 2).

Table 2. *Amounts of phosphorus released in* 7 *min.* (P_7) *at* 100° *in* N-HCl *from the barium-insoluble fractions of TCA extracts prepared from livers of control guinea pigs and of guinea pigs treated with carbon tetrachloride*

Control animals				Animals treated with carbon tetrachloride			
Fat (%)	N (%)	P_7 (mg./100 g.)	P_7 (mg./g. N)	Fat (%)	N (%)	P_7 (mg./100 g.)	P_7 (mg./g. N)
—	3·89	4·8	1·2	23·0	2·15	9·8	4·5
—	3·58	6·4	1·7	10·8	2·53	5·5	2·1
—	3·63	3·9	1·1	15·9	1·86	6·9	3·7
—	3·25	3·3	1·0	13·0	1·77	5·3	3·0
—	3·57	3·8	1·1	16·2	1·84	6·1	3·3
9·8	3·48	4·7	1·4	12·5	2·20	6·2	2·8
—	3·51	5·7	1·6	13·4	2·79	5·9	2·1
6·6	3·58	1·9	0·6	14·5	1·95	8·8	4·5
6·6	3·73	4·5	1·1	10·6	2·46	6·9	2·9
6·0	3·06	6·3	2·0	13·0	2·30	8·0	3·6
5·4	3·65	7·4	2·0	14·6	2·20	8·5	3·9
9·0	3·57	7·5	2·1	—	—	—	—
8·8	3·93	8·3	2·1	—	—	—	—
		Mean 5·3	1·4			7·1	3·3

Most workers have been unconcerned with the origin of the inorganic phosphate released from this fraction in 7 min. at 100° in N-acid and many have, in fact, referred to it as *ATP*. In muscle extracts this phosphate certainly arises from *ATP*, but the picture in liver is somewhat different, as Kalckar (1945) has pointed out. Kaplan & Greenberg (1944*c*) concluded that the compound is *ATP*, but their analytical methods have been criticized by Kalckar (1945), although it is not clear just how his criticism can be applied to the results obtained by these workers. A more satisfactory method for the identification of the compound responsible for the liberation of the phosphate under the conditions of hydrolysis lies in the determination of the N, P and pentose ratio. By this method Le Page & Umbreit (1945) and Le Page (1946) found that in the livers of rats the ratio of *ADP* to *ATP* is about 17 : 1. On the other hand Rapoport (1945), using myosin and a specific nucleotidase, produced evidence indicating a ratio of 2 : 1 in the fed, and 5 : 1 in the starved, liver.

We have also determined the ratios of N : 7 min.-labile P : pentose in the reprecipitated barium insoluble fractions. The normal liver gave a ratio of 5·0 : 1·3 : 0·9 and the fatty liver 5·0 : 1·3 : 1·0, and since *ADP* requires a ratio of 5·0 : 1·0 : 1·0 it might be concluded that a considerable proportion of the P released is derived from *ADP*. This conclusion, however, was not confirmed when the adenosine triphosphatase (*ATP*-ase) method (Rapoport & Nelson, 1945) for the determination of *ATP* was employed. From the N, P and pentose determinations a molar ratio of *ATP* to *ADP* ranging from 1 : 1 to 1 : 7 was indicated, whereas the ratios as determined by the myosin *ATP*-ase ranged from >90 : 1 to 2 : 1. Because of the high degree of substrate specificity of the *ATP*-ase it would seem that these figures are the more reliable. The explanation of these differences lies in the possibility of contamination of the fraction with some N compound, the presence of which will greatly affect the ratio of labile P to N.

Having established that there was a marked increase in the 7 min.-hydrolyzable fraction, it was considered desirable to investigate the possibility of changes in the concentration of compounds hydrolyzable at longer intervals. Hydrolysis curves were determined on samples of both the original *TCA* extracts and the barium-insoluble fraction. All hydrolyses were carried out in N-HCl at 100° for periods of 7, 15, 30, 60 and 180 min., and on removal from the bath the tubes were rapidly cooled in a freezing mixture and then analyzed for the inorganic phosphate released. Figs. 1 and 2 illustrate curves typical of those obtained from normal and fatty livers. It will be seen that the general shape of the curves is similar, but those illustrating the behaviour of the phosphate compounds in the fatty liver are on a higher level, due largely to the presence of increased amounts of compounds hydrolyzable in 7 min. This is more clearly seen from the data given in Table 3. At the 7–15 min. interval in the *TCA* extracts the difference in favour of the fatty liver is proportionally the same as in the 0–7 min. interval. For the remaining intervals no certain conclusions can be reached as to any differences between the normal and fatty liver.

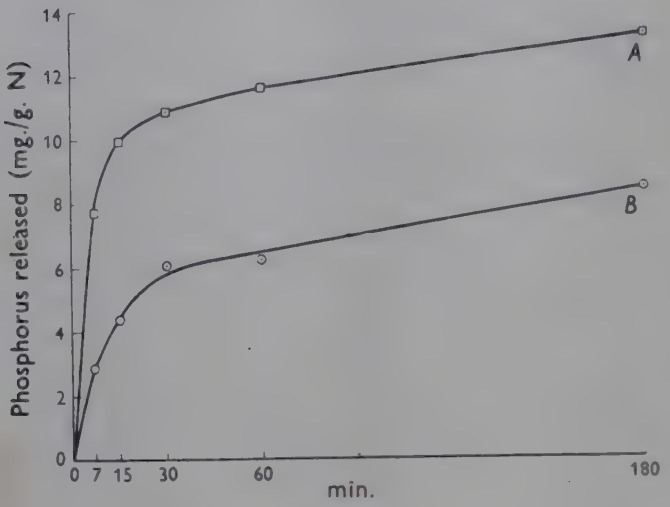

Fig. 1. Hydrolysis curves illustrating the amounts of inorganic P released at 100° in N-HCl for varying periods from *TCA* extracts of a fatty liver (*A*) and a normal liver (*B*). All points on the curves represent the means of at least two determinations.

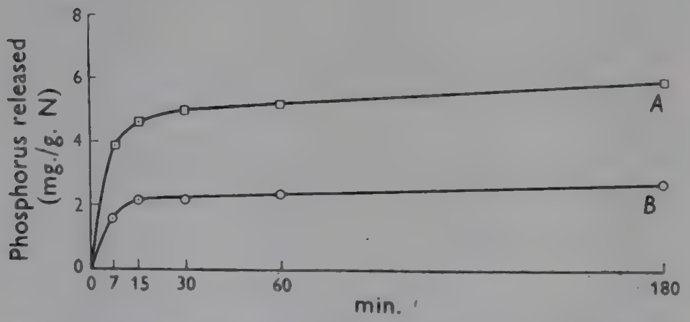

Fig. 2. Hydrolysis curves illustrating the amounts of inorganic P released at 100° in N-HCl for varying periods from the barium-precipitable fraction of *TCA* extracts, the hydrolysis curves of which are shown in Fig. 1. *A*, fatty liver, *B*, normal liver. All points represent the means of at least two determinations.

The compounds which appear in the *TCA* extracts of liver include *ATP*, *ADP*, glucose-1-phosphate, phosphocreatine, phosphopyruvic acid, hexosediphosphate, fructose-6-phosphate, phosphoglyceric acid and possibly acetyl phosphate. Breakdown of these compounds, if present in our extracts, must therefore be responsible for the differences between the normal and the fatty liver in the 0–7 min. period. Since the first four compounds and acetyl phosphate are completely hydrolyzed in this interval, they are likely to be responsible for by far the greater part of the P released.

ATP, *ADP* and hexosediphosphate are precipitable by barium and it follows that the differences between the amounts of P released in 7 min. in the *TCA* extracts and in the barium-insoluble fraction will show whether the increased amount of *APP* is, in fact, entirely responsible for the difference noted in the 7 min.-hydrolyzable compounds present in the *TCA* extract. These differences, together with those at other intervals, are presented in Table 4, and in most instances were obtained by direct estimation in the barium-soluble fraction and not from the difference between the values for the *TCA* extracts and the barium-insoluble fraction.

These figures also show a marked difference between the normal and fatty livers, and it is clear that the increased amount of P released in 7 min. from *TCA* extracts of fatty livers (Table 2) is not entirely due to the differences which have been noted in the barium-precipitable fraction. Thus, in addition to a rise in the *APP* content, there is also an increase in readily hydrolyzable compounds in the barium soluble fraction. These differences persist to about the same degree over the 7–15 min. interval, whilst the amounts released over the 15–30 min. period are of the same order. Rather more P is apparently released between 30 and 60 min. with the fatty livers, but here, as in the next interval, the results are too variable to permit any definite conclusions. No attempt has been made, as yet, to identify the compounds responsible for any differences noted at the longer time intervals since interest was, in the main, centred on the more readily hydrolyzable compounds.

Most workers, who have been concerned with the estimation and the identification of labile P compounds, appear to have used either the method of Fiske & Subbarow (1925) or modifications thereof. In these methods the phosphomolybdate is reduced in aqueous solution and it is essential to adhere strictly to carefully standardized conditions in order to achieve consistent and dependable results (cf. Stone, 1943).

Table 3. *Amounts of phosphorus released at various time intervals in* N-HCl *at* 100° *from TCA extracts and barium-insoluble fractions prepared from livers of control guinea pigs and of guinea pigs treated with carbon tetrachloride*

	0–7 min.		7–15 min.		15–30 min.		30–60 min.		60–180 min.		Total, 180 min.	
	(mg./g. N)	(mg./100 g.)	(mg./g. N)	(mg./100 g.)	(mg./g. N)	(mg./100 g.)	(mg./g. N)	(mg./100 g.)	(mg./g. N)	(mg./100 g.)	(mg./g. N)	(mg./100 g.)
TCA EXTRACT												
Animals treated with carbon tetrachloride												
	7·8	17·9	—	—	—	—	—	—	—	—	—	—
	6·2	14·7	—	—	—	—	—	—	—	—	—	—
	6·3	11·6	2·8	5·3	1·4	2·6	1·0	1·9	0·8	1·4	13·1	24·2
	5·5	11·9	2·4	5·3	1·0	2·3	0·7	1·6	1·5	3·7	10·6	23·4
	5·0	13·2	2·1	5·8	0·5	1·4	0·9	2·7	—	—	—	—
	9·6	18·6	3·2	5·6	1·1	2·2	1·0	1·7	1·7	3·5	15·3	29·8
	7·8	17·2	2·2	4·7	0·9	1·9	0·7	1·6	1·5	3·5	14·3	31·5
Mean	**6·9**	**15·0**	**2·5**	**5·3**	**1·0**	**2·1**	**0·9**	**1·9**	**1·4**	**3·0**	**13·3**	**27·2**
Control animals												
	2·9	10·4	1·5	5·4	1·7	5·8	0·0	0·0	2·2	8·0	9·5	35·3
	3·9	14·3	1·1	4·1	1·0	3·6	0·1	0·2	1·9	7·0	11·1	31·4
	4·2	14·9	1·4	5·0	0·5	1·8	0·0	0·0	2·2	7·8	12·0	43·1
	4·0	15·8	1·2	4·9	0·8	3·0	1·2	4·6	1·1	4·7	10·0	39·4
	2·4	8·6	—	—	—	—	—	—	—	—	—	—
	3·0	11·8	—	—	—	—	—	—	—	—	—	—
	4·0	14·3	—	—	—	—	—	—	—	—	—	—
	3·0	11·0	—	—	—	—	—	—	—	—	—	—
	4·5	15·7	1·6	5·6	0·3	1·3	1·2	5·2	—	—	—	—
Mean	**3·5**	**13·0**	**1·4**	**5·0**	—	—	—	—	—	—	—	—
BARIUM-INSOLUBLE FRACTION												
Animals treated with carbon tetrachloride												
	2·6	8·0	—	—	—	—	—	—	—	—	—	—
	2·9	6·9	—	—	—	—	—	—	—	—	—	—
	3·3	6·1	0·6	1·4	0·6	1·0	0·2	0·3	0·5	1·0	3·1	5·6
	2·8	6·2	0·7	1·5	0·2	0·3	0·2	0·6	0·5	0·8	2·9	6·6
	2·1	5·9	0·8	2·4	0·4	1·0	0·0	0·0	—	—	—	—
	4·5	8·8	1·4	2·6	0·5	1·0	0·5	0·9	1·0	2·0	1·6	3·2
	3·9	8·5	0·7	1·7	0·4	0·8	0·2	0·3	0·7	1·6	3·0	6·6
Mean	**3·2**	**7·2**	**0·8**	**1·9**	—	—	—	—	—	—	—	—
Control animals												
	1·6	5·6	0·6	1·9	0·0	0·0	0·1	0·3	0·4	1·4	1·8	6·3
	2·0	7·4	0·5	1·9	0·4	1·6	0·0	0·0	0·9	3·3	1·8	6·4
	2·1	7·5	0·6	2·0	0·3	1·1	0·0	0·0	0·4	1·4	2·8	9·8
	2·1	8·3	0·6	2·3	0·1	0·2	0·7	2·6	0·2	0·8	1·8	7·1
Mean	**1·9**	**7·2**	**0·6**	**2·0**	—	—	—	—	—	—	—	—

The presence of phosphocreatine in rat-liver extracts has been denied (Flock *et al.* 1936*a*) and affirmed (Le Page & Umbreit, 1945; Le Page, 1946), although in no case have any specific methods for its identification been employed. The failure of Flock *et al.* (1936*a*) to detect phosphocreatine may have been due to their use of mercury for the removal of glycogen from the extracts. This metal is known to produce rapid catalytic decomposition of phosphocreatine (Fiske & Subbarow, 1929). Since the latter is very labile in acid molybdate, it is customary to follow either the comparative colorimetric procedure employed by Fiske & Subbarow (1929), or to precipitate the inorganic phosphate and then obtain the phosphocreatine by difference. A somewhat similar procedure was adopted in the first instance for the determination of acetyl phosphate (Lipmann & Tuttle, 1944). In the method for the determination of P devised by Berenblum & Chain (1938), however, the acid molybdate-labile compounds, if present, are only in contact with the acid phase for 1 min. during the extraction of the phosphomolybdate complex by *iso*butanol. The aqueous layer is then discarded and it is thus only the P released in this short interval which adds to the 'true' inorganic phosphate present. According to Fiske & Subbarow (1929) 28·9 % of the phosphocreatine present is hydrolyzed in 3 min. at 18° in acid molybdate and Lipmann & Tuttle (1944) have shown that acetyl phosphate is 75 % decomposed in *c.* 2 min. at 23°.

Table 4. *Amounts of phosphorus (mg./g.* N*) released in the indicated intervals from compounds present in the barium-soluble fraction of TCA extracts of livers of control guinea pigs and of guinea pigs treated with carbon tetrachloride, and hydrolyzable in* N-HCl *at* 100°

0–7 min.	7–15 min.	15–30 min.	30–60 min.	60–180 min.	Total, 180 min.
Animals treated with carbon tetrachloride					
3·0	2·2	0·8	0·8	0·3	10·0
2·7	1·7	0·8	0·5	1·0	7·7
2·9	1·3	0·1	0·9	—	—
5·1	1·8	0·6	0·5	1·2	13·2
3·9	1·5	0·5	0·5	0·8	11·0
3·3	—	—	—	—	—
5·2	—	—	—	—	—
Mean 3·7	1·7	—	—	—	—
Control animals					
1·3	0·9	1·7	0·0	1·8	7·7
1·9	0·6	1·0	0·0	1·0	9·0
2·1	0·8	0·2	0·0	1·8	9·4
1·9	0·6	0·8	0·5	1·9	8·2
1·8	—	—	—	—	—
1·9	—	—	—	—	—
2·0	—	—	—	—	—
Mean 1·8	0·7	—	—	—	—

It is thus clear that these compounds will only be estimated in part as inorganic phosphate when the Berenblum & Chain (1938) method is employed, and the remainder would be estimated together with other labile compounds in the 7 min.-hydrolyzable fraction. The compounds which are likely to make a substantial contribution to the amount of P released in 7 min. in the barium-soluble fraction are phosphocreatine, glucose-1-phosphate, phosphopyruvic acid and possibly acetyl phosphate. It follows, therefore, that if these are present in increased amounts in the fatty liver, they would account in some measure for the increase in the 7 min.-hydrolyzable material seen in the barium-soluble salts derived from the *TCA* extracts of the fatty liver.

The presence of a molybdate-labile compound or compounds in the *TCA* extracts was indicated by increases in the amounts of P measurable after allowing the sample to remain in the acid molybdate for 30 min. at 30° before extraction of the phosphomolybdate complex with *iso*butanol. Under these conditions we have established that phosphocreatine is completely decomposed and that no P is split from *ADP* or *ATP*.

The results (Table 5) indicate that there exists a marked difference between the control and the treated series and that, moreover, the normal liver contains some acid molybdate-labile compound or compounds. Since the fatty livers from guinea pigs treated with carbon tetrachloride exhibit an increased acetoacetic acid production (Ennor, 1942), it may be presumed that increased amounts of fat are being oxidized. It would seem, therefore, that such livers should contain demonstrable amounts of acyl phosphates, if these are formed from the fatty acid chain as has been suggested by Lehninger (1945*b*). We have made repeated attempts to detect the presence of this compound by the hydroxylamine method described by Lipmann & Tuttle (1945). In no case has a positive reaction been obtained, even though extracts have been employed containing amounts of labile P corresponding to 60 μg. of acetyl phosphate/ml.

Table 5. *Acid molybdate-labile compounds present in TCA extracts of livers from control guinea pigs and from guinea pigs treated with carbon tetrachloride*

Control animals		Animals treated with carbon tetrachloride	
P (mg./g. N)	P (mg./100 g.)	P (mg./g. N)	P (mg./100 g.)
0·9	2·7	—	—
0·2	0·6	1·5	3·1
0·4	1·4	1·7	2·8
0·8	2·8	2·1	4·1
0·2	0·7	1·8	4·8
0·5	1·6	—	—
Mean 0·5	1·6	1·8	3·7

The possibility of the presence in the extracts of substances which might interfere with the reaction has been checked by the detection of added succinylhydroxamic acid corresponding to 25 μg. of acetylphosphate.

It appears, therefore, that the suggestion of Kaplan & Greenberg (1944*c*) that the acid molybdate-labile compound present in their extracts was acetyl phosphate is unlikely. It may also be noted that Kaplan & Greenberg based their suggestion upon the premise that phosphocreatine was not present in the liver. This is in disagreement with the results of Le Page & Umbreit (1945), although these latter workers did not provide unequivocal proof that the labile compound was phosphocreatine. We have tried to obtain more certain evidence by correlating the labile P with the bound creatine. Such a correlation has been achieved with extracts from both normal and fatty livers, but because of the presence of inhibitors which interfere with the estimation of free creatine, the analytical methods are being investigated. It is hoped that the results will be published later. For the moment, however, from these unpublished experiments, it is considered that the acid molybdate-labile P arises for the most part from phosphocreatine, and certainly not from acetyl phosphate.

DISCUSSION

Since the technique adopted throughout these investigations differs, in some respects, from that of other workers it is desirable to point out these differences and to discuss any bearing they may have on the results. It is generally accepted that true, normal 'resting values' for lactic acid and *ATP* in brain and muscle will not be obtained unless the organs are removed under anaesthesia. We have not used any anaesthetic before removal of the liver and have found no unequivocal evidence in the literature that anaesthesia is necessary to obtain maximum *ATP* and *ADP* levels in this organ. Le Page (1946), however, has found some indication of a loss of rat-liver *ATP* when the whole animal was frozen without anaesthesia. To detect any changes in the absence of anaesthesia we have taken a group of six animals, three of which were treated with carbon tetrachloride. All were given an injection of nembutal (50 mg./kg. body wt.) and, when light surgical anaesthesia had been effected, the liver was rapidly removed and dropped into liquid air. In all cases the *TCA* extracts and the corresponding barium-insoluble fractions gave analytical figures which confirmed the previous findings; hence no decomposition of the labile phosphate detectable by our methods occurs in the liver of guinea pigs when the animals are killed without anaesthesia.

The estimation of the N : 7 min.-labile P : pentose ratios has been carried out by Le Page & Umbreit (1945) and Le Page (1946) to establish the identity of the adenosine polyphosphates in the barium-insoluble fraction. Our results, with similar methods, on guinea-pig liver, confirm the opinions of these workers and indicate that a considerable fraction of the 7 min.-labile P has its origin in *ADP*. This conclusion is not supported by results obtained with the *ATP*-ase method. These were only few in number since we were concerned initially with quantitative changes in those compounds possessing high-energy phosphate bonds ($\sim$ph; Lipmann, 1941). As far as the determination of the identity of the compounds is concerned, it seems more likely that dependable results will be achieved with the specific *ATP*-ase than with chemical methods, which will be subject to error from possible contamination with N or P compounds. It is worthy of note that Rapoport (1945), using the *ATP*-ase method, has found *ATP* in livers of rats in amounts much greater than those found by Le Page & Umbreit (1945) and Le Page (1946).

The changes in the distribution of the acid-soluble phosphates in the fatty liver of the guinea pig, in so far as we have investigated them, indicate a marked increase in *APP* and phosphocreatine. In the interpretation of these results the work of Kaplan & Greenberg (1944*b*) is of interest, as these workers, using fasted and fat-fed rats, claimed a decrease in $\sim$ph levels, attributed to the appropriation of $\sim$ph for the oxidation of fat without a replacement being effected through the utilization of acetoacetate in the liver. It is doubtful, however, if such a hypothesis is tenable, since it assumes the obligatory formation of acetoacetate as the end product of fatty acid oxidation. This is not necessarily so as is indicated by numerous experiments with anti-ketogenic substances. An explanation for the non-appearance of acetoacetate may be found in the association of the citric acid cycle with fatty acid oxidation (cf. Quastel & Wheatley, 1935). Positive evidence for this is found in the action of the enzyme citrogenase, present in the liver (Breusch, 1943), which catalyzes the reaction between acetoacetate and oxaloacetate to form citrate (Wieland & Rosenthal, 1943; Buchanan, Sakami, Gurin & Wilson, 1945 *a*, *b*).

It is therefore suggested that the increased amount of $\sim$ph which has been noted in our experiments arises from energy derived from the citric cycle as a direct consequence of the increased amounts of fat which are being oxidized.

It has been suggested by Lehninger (1945*b*) that $\sim$ph might be generated by the stepwise oxidation of fatty acids down to the stage of acetoacetate. Experiments with malonate suggest that this process is unlikely to result in increases in $\sim$ph. Thus the well known *in vivo* and *in vitro* effect of malonate in increasing acetoacetate production is generally assumed to be due to the denial of the citric acid cycle as one avenue for the oxidation of fat. Moreover, malonate has been shown by Kaplan & Greenberg (1944*a*) to produce a decrease in $\sim$ph in the rat liver. It would seem, therefore, that the increases in $\sim$ph which we have observed are due not to the reactions involved in the degradation of the fat down to the acetoacetate level, but to the reactions which occur following the entry of the acetoacetate into the citric acid cycle.

The increased amount of *APP* which we have observed in the fatty livers of guinea pigs is of interest in view of the work of Lehninger (1945*b*) on the role of *ATP* and *ADP* in fatty acid oxidation. In considering the implication of these results it should be remembered that Lehninger showed that, in crude rat-liver homogenates, the activation produced by *ADP* was equal to that by *ATP* when both were present in equimolar concentrations. Under these conditions there is twice the amount of ~ph available in the *ATP*-catalyzed system as in that catalyzed by *ADP*. When the molar concentration of *ATP* was reduced to one half that of *ADP*, thus providing equal amounts of ~ph, *ATP* produced only 70% of the activation provided by *ADP*. It does not seem that this can be explained by the assumption that the diphosphate is raised to the triphosphate stage by phosphorylation, since the addition of adenylic acid, which presumably would be phosphorylated if this were so, was without effect. The only conclusion to be drawn from these results is that *ADP* is at least as important as *ATP* to the *in vivo* systems responsible for the oxidation of fatty acids.

In later work Lehninger (1945*a*) employed purified homogenates and showed that although *ATP* produced the customary activation, *ADP* was inactive. Lehninger interpreted this as indicating the specific nature of the activation produced by *ATP*. While this is undoubtedly so in the system employed, the earlier results (Lehninger, 1945*b*) cannot be discounted. They suggest that, whilst the study of purified liver homogenates will undoubtedly give valuable evidence as to the precise nature of the activators of a particular system, the findings are not necessarily applicable without modification to the oxidation of fatty acids in the intact liver occurring in association with the oxidation of other substrates.

On thermodynamic grounds, the phosphorylation of *ADP* to *ATP* requires no greater utilization of energy than that of creatine to phosphocreatine. But it is of interest that the fatty liver, whilst containing greater amounts of ~ph (*APP* and phosphocreatine), does not contain exclusively *ATP* if complete reliance be placed upon the determination of the N : 7 min.-labile P : pentose ratios. Some doubt as to the reliability of these analyses arises when one considers the increases in phosphocreatine which have been demonstrated, since it is difficult to conceive of ~ph being transferred to creatine without a prior transfer to saturation point of the adenylic acid system, and with the consequent appearance of *ATP*. Further experiments with the more specific *ATP*-ase methods should settle this point.

It is well known that phosphocreatine is capable of phosphorylating adenylic acid, and Lehninger (1945*b*) has shown that the activation of fatty acid oxidation is greater when both adenylic acid and phosphocreatine are present than with phosphocreatine alone. The phosphocreatine found in the normal liver may then be regarded as representing a readily available source of energy, which can be utilized through the adenylic acid system when the occasion arises, and its presence in increased amounts in the fatty liver is most probably the result of an overflow of ~ph which cannot be stored as *APP*.

The continued failure to detect the presence of acyl phosphates in the fatty liver is disappointing in view of the accumulated evidence which suggests the necessity of phosphorylation as an integral part of the fatty acid oxidation mechanism. That acyl phosphates may be oxidized in the absence of added *ADP* or *ATP* (Lehninger, 1944) is interesting but is not conclusive proof of the intermediate formation of these compounds, since they themselves are capable of phosphorylating adenylic acid with the formation of *ATP*.

SUMMARY

1. The change in the distribution of acid-soluble phosphates in the fatty livers of guinea pigs treated with carbon tetrachloride has been investigated.

2. In such livers there is an increase in the total acid-soluble organic phosphates.

3. This increase is considered as primarily due to increases in adenosine polyphosphates and phosphocreatine, and in general runs parallel to the fat content.

4. Acyl phosphates have not been detected in liver extracts.

5. The results are interpreted in the light of modern work on fatty acid oxidation.

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The Estimation of Creatine

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During the course of an investigation into the metabolism of fatty livers (Ennor & Stocken, 1948) it became desirable to identify, and subsequently to estimate, amounts of a very labile phosphorus compound present in the livers of normal guinea pigs and in others treated with carbon tetrachloride. Although phosphate liberated from organic phosphate compounds in acid molybdate is often considered to arise from phosphocreatine, there are other possibilities, and we endeavoured therefore to correlate the labile phosphorus with the creatine liberated following mild hydrolysis. It was decided to estimate the creatine as such rather than after conversion to creatinine, and an attempt was made to employ the method of Eggleton, Elsden & Gough (1943). These authors have pointed out that trichloroacetic acid (*TCA*) extracts of liver contain inhibitory substances which prevent the estimation of creatine by the Barritt (1936) modification of the Vosges & Proskauer (1898) reaction. The suggestion was also made that these inhibitors were the ω-amino aliphatic acids.

In the fractionation of *TCA* extracts of guinea-pig liver by means of barium (Le Page & Umbreit, 1945) it was found that both the inhibitor and phosphocreatine were precipitated in the ethanol-insoluble fraction. An examination of this fraction indicated that the inhibitory substance or substances was more likely to be glutathione than an amino-acid as suggested by Eggleton *et al.* (1943). Such a supposition, because of the wide distribution of the tripeptide, necessarily casts some doubt upon the validity of the application of the α-naphthol—diacetyl reaction to the estimation of creatine in tissues.

This communication is concerned with some experiments on the inhibition of the Barritt reaction by sulphydryl compounds and suggests a modification of the procedure of Eggleton *et al.* by means of which a more accurate determination of the creatine content of tissues may be made.

EXPERIMENTAL

Reagents

Creatine hydrate. An A.R. specimen was recrystallized according to Hunter (1928) and its purity checked by N analysis.

Diacetyl. A stock solution of approx. 1 % was prepared from A.R. dimethylglyoxime as described by Walpole (1911). This was diluted and used as described by Eggleton *et al.* (1943). No apparent loss in activity has been observed following 3 months' storage at −1°.

α-Naphthol. A faintly coloured A.R. sample has been used throughout. It was considered unnecessary to effect further purification by steam distillation. The solution of this compound and the volume used was, in all cases, as described by Eggleton *et al.* (1943).

Glutathione (*GSH*). This was a commercial specimen.

Oxidized glutathione (*GSSG*). This was prepared by bubbling O_2 through a slightly alkaline solution of GSH of appropriate concentration until a negative nitroprusside reaction was obtained.

Benzyl mercaptan and methionine. These were gifts from the Ministry of Supply.

Cysteine ester hydrochloride. This compound was mentioned casually by Friedmann (1903) who gave no details of the synthesis. A mixture of 20 g. cystine, 60 ml. conc. HCl (36·5 % w/v), 20 ml. water, 200 ml. ethanol and 20 g. tin foil were gently refluxed until all the tin had dissolved. After removal of the tin with H_2S, the solution was evaporated to dryness *in vacuo* and the residue dissolved in 150 ml. ethanol and saturated with dry HCl at 0°. Crystals of cysteine ester hydrochloride separated on standing overnight. Yield 24 g. (79 %). M.p. 128°. SH by I_2 titration, 17·7 %; calc. for $C_5H_{12}O_2SNCl$, 17·8 %.

Cystine ester hydrochloride. This was prepared according to the method of Friedmann (1903).

p-Chloromercuribenzoic acid was synthesized according to Whitmore & Woodward (1932).

2:3-*Dimercaptopropanol* (*BAL*) was obtained as described by Stocken (1947).

Procedure

This, apart from one minor modification, has followed the method described by Eggleton *et al.* (1943). Greater reproducibility is obtained if, after adjusting the volume of the creatine-α-naphthol-diacetyl solution to 10 ml. in a narrow tube, the contents are transferred to a 6 × 1 in. tube for mixing and development of the colour. After 30 min. development at room temperature (18°) the intensity of the colour was measured in 1 cm. cells in the Hilger Spekker absorptiometer with Ilford filter no. 604 (spectrum green).

Influence of monothiols on the Barritt reaction

Cysteine ester hydrochloride. For preliminary tests and because of the small amount of GSH available, the initial experiments were carried out with cysteine ester hydrochloride. Varying amounts of this compound were added to tubes each containing 1·0 ml. of 5×10^{-4}M-creatine (65·5 μg.). The percentage inhibition of colour development was then calculated and plotted against the molar ratio, cysteine ester : creatine.

The influence of cysteine ester on the colour produced by creatine, diacetyl and α-naphthol is very marked, 60 % inhibition being observed with a molar ratio of cysteine ester : creatine of 0·8 : 1.

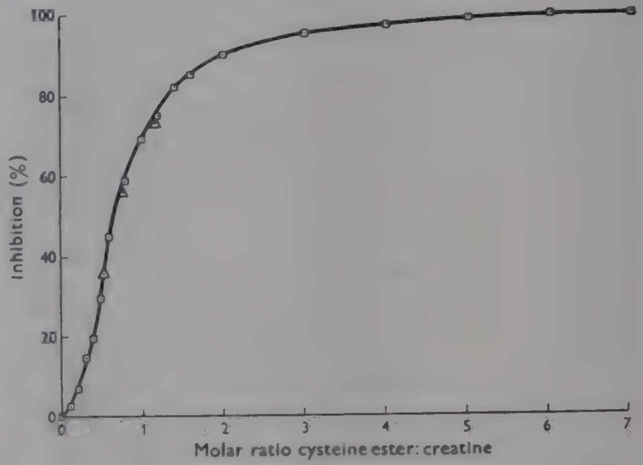

Fig. 1. Inhibition of the Barritt reaction by varying amounts of cysteine ester hydrochloride and glutathione. 65·5 μg. of creatine throughout. To all tubes were added 2·0 ml. α-naphthol solution (1·0 g. α-naphthol in 100 ml. of a solution containing 6·0 g. NaOH + 16 g. Na_2CO_3) + 1·0 ml. of a 1 in 20 dilution of stock diacetyl + varying amounts of thiol. Adjusted to 10 ml. with water and colour measured after 30 min. at room temperature. ⊡ denotes cysteine ester hydrochloride; △ denotes glutathione.

Glutathione (*GSH*). To test the inhibitory effect of GSH three points on the cysteine ester curve were chosen and the colour produced by creatine in the presence of a GSH : creatine ratio of 0·5, 0·8 and 1·2 mol. to 1 was determined. The points marked △ in Fig. 1 were obtained in this manner and are sufficiently close to the cysteine-ester inhibition curve to suggest that it is primarily the thiol group which is responsible for the inhibition of the Barritt reaction.

Benzyl mercaptan and 2:3-*dimercaptopropanol.* Two simple thiols were used in an attempt to show that the thiol group is the determining factor in the inhibition of the colour.

Table 1. *Inhibition of Barritt reaction by simple thiols*

	Inhibition (%)	
Molar ratio thiol : creatine	Benzyl mercaptan	BAL
0·5	47	11·5
0·8	66	17·5
1·2	84·5	24·0
2·0	—	46·5

The results (Table 1) obtained with benzyl mercaptan are similar to those found with cysteine ester and GSH and support the idea that the inhibition seen with the latter compounds is due primarily to the reactivity of the thiol group and not to the amino group. Further confirmation of this is given in the section dealing with the neutralization of the inhibitor. It will be noticed that the degree of inhibition given by BAL is very much less than that produced by the other compounds. It is likely that the inhibition is due to addition of the thiol group across some reactive double bond formed in the sequence of reactions leading to the coloured complex. If this is the case, it is not to be expected that BAL would be as efficient an inhibitor as benzyl mercaptan even if it be supposed that only one of the thiol groups in BAL is capable of such an addition.

It is clear from the foregoing experiments that thiol-containing compounds present in tissues will produce considerable interference with the estimation of creatine, and that the magnitude of the error introduced will depend on the ratio of thiol to creatine. Thus, in those tissues where the creatine content is low and the thiol content high, it would be anticipated that the method discussed would not even detect creatine.

Effect of other sulphur-containing compounds

It was considered unlikely that the oxidation of the SH group would eliminate the inhibition because of the well known tendency towards cleavage of the disulphide linkage in alkaline solution. Experiments with GSSG and cystine ester (Table 2) confirm this, although it will be noticed that GSSG produces a much greater degree of inhibition, and is apparently, therefore, much more susceptible to hydrolysis than is cystine ester.

Table 2. *Disulphide inhibition of the Barritt reaction*

Molar ratio disulphide : creatine	Inhibition (%) Cystine ester	GSSG
0·25	—	22·0
0·4	—	34·0
0·6	—	50·0
1·0	3·4	—
2·0	5·0	81·0
4·0	9·1	—
8·0	18·1	—
16·0	31·9	—

It is clear from these experiments that not only thiols, but any compound which generates a thiol on treatment with alkali, will seriously interfere with the estimation of creatine in tissue extracts. It is to be regretted that the only other sulphur compound of biological importance which we have had the opportunity of testing in this reaction was methionine. This produced an inhibition of about 5% when present in a molar ratio of 4 methionine : 1 creatine.

Protection of the Barritt reaction from thiol inhibition

It is apparent that when the molar ratio of SH to creatine exceeds 0·2–0·4 (7–15% inhibition), the successful application of the Barritt reaction will depend on the removal of SH groups. Compounds known to combine with thiol groups include iodoacetic acid, maleate, trivalent arsenicals and organic mercurials. Of these we preferred the mercurials because of the high rate of formation and stability of the resultant R-Hg-SR' compound in alkaline solution. p-Chloromercuribenzoic acid was chosen as a possible protective agent in order to ensure non-precipitation of the Hg derivative.

Procedure. 1·0 ml. of a 5×10^{-4} M-solution of creatine (65·5 μg.) was added to a series of tubes, followed by 1·0 ml. of 4×10^{-3} M-cysteine ester. This gave an initial ratio of cysteine ester : creatine of 8 : 1 (a ratio of 7 : 1 will produce 100% inhibition under these conditions, see Fig. 1). Varying volumes of an appropriate dilution of a 10^{-2} M-solution of p-chloromercuribenzoic acid (*CMBA*) were then added to produce *CMBA* : cysteine ester ratios from 0 to 2·0. α-Naphthol and diacetyl were then added as before and the resultant colours estimated after 30 min. All determinations were carried out in duplicate and the figures in Table 3 are the means of the values obtained.

Table 3. *Protection afforded by p-chloromercuribenzoic acid (CMBA) on the thiol inhibition of the Barritt reaction*

(Degree of protection = μg. creatine found/μg. creatine present × 100.)

Molar ratio *CMBA* : cysteine ester	Degree of protection
0·1	0
0·2	1·5
0·3	2·0
0·4	2·8
0·5	2·9
0·6	5·4
0·7	11·8
0·8	31·5
0·9	73·7
1·0	89·6
2·0	92·2

Increasing amounts of *CMBA*, as would be expected, lead to an increased removal of the inhibition produced by cysteine ester (Table 3). Complete removal of the inhibition, however, was not obtained even when the molar ratio of *CMBA* : cysteine ester was 2 : 1. Any of the three following factors could be responsible for this effect: (*a*) dissociation of the resultant R-Hg-SR', (*b*) the R-Hg-SR' itself, (*c*) the excess of the *CMBA*.

The degree of protection afforded by *CMBA* against the effects of GSH, GSSG, benzyl mercaptan and BAL was similar to that obtained in the case of cysteine ester.

Effect of ethyl α-amino-β-(p-carboxyphenylmercurithio)-propionate and p-chloromercuribenzoic acid on the Barritt reaction. The ethyl α-amino-β-(*p*-carboxyphenylmercurithio)-propionate was not isolated, but prepared in solution by mixing equivalent amounts of *p*-chloromercuribenzoic acid and cysteine ester. Convenient amounts of suitable dilutions were then added to 1·0 ml. 5×10^{-4}M-creatine (65·5 μg.). The subsequent procedure was as described earlier.

Table 4. *Inhibition of the Barritt reaction by p-chloromercuribenzoic acid (CMBA) and ethyl α-amino-β-(p-carboxyphenylmercurithio)-propionate* (*R*-Hg-S*R′*)

Molar ratio compound : creatine	Inhibition (%)	
	R-Hg-S*R′*	*CMBA*
0·5	0	0
1·0	0	0
2·0	0	1·7
4·0	4·9	4·5
8·0	5·3	6·1
10·0	—	7·4
16·0	8·2	—
32·0	11·1	—

The results of this experiment (Table 4) indicate that some inhibition is to be expected when the ratio of either the complex, or of the *CMBA*, to creatine exceeds 2 : 1. Since, however, a ratio of 4 : 1 only produces an inhibition of 5 % and a ratio of 2 mol. *CMBA* to 1 mol. cysteine affords 92 % protection, it is clear that the error introduced is small compared with that obtained when free thiol compounds are present.

Stoicheiometric relationship of the cysteine ester inhibition. It was considered worth while to determine the colour developed by varying amounts of creatine in the presence of varying amounts of cysteine ester when the relative amounts of each were maintained at a constant ratio. A molar ratio of cysteine ester to creatine of 0·6 : 1 was chosen since at this value the inhibition expected is 44·8 % (Fig. 1).

The strictly linear relationship between the amount of creatine and the optical density is shown in Fig. 2. In the presence of cysteine it will be noticed that there is a distinct diminution in the inhibition at low concentrations of the thiol (Fig. 3).

If it is true that interference with the production of the coloured compound is due to a preferential reaction of some intermediate compound with the thiol then it would be expected, under the conditions of this experiment, that the percentage inhibition would remain constant. This, however, is not the case, and the explanation is probably to be found in the small absolute amount of cysteine ester which is present at the lower creatine levels. Thus at 8·18 μg. of creatine there are only 5·6 μg. of cysteine ester in a volume of 10·0 ml., and it must be considered unlikely that such a small amount would remain in the reduced state. The actual ratio of cysteine ester to creatine would thus be less than 0·6 : 1. Reference to Fig. 1 shows that a reduction of this ratio to 0·4 : 1 would account for the low inhibition seen at the 8·18 μg. level of creatine.

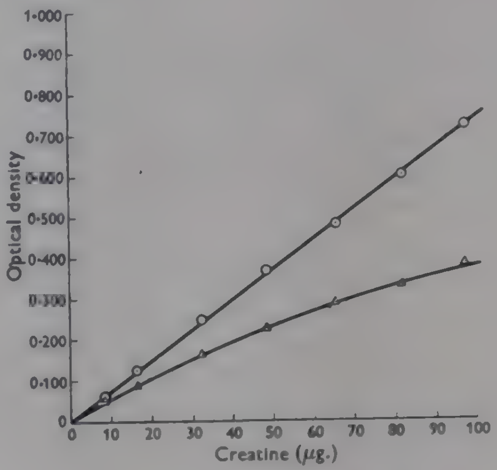

Fig. 2. Comparison of the colour produced by varying amounts of creatine in the absence ⊙ and the presence △ of a molar ratio of cysteine ester : creatine of 0·6 : 1.

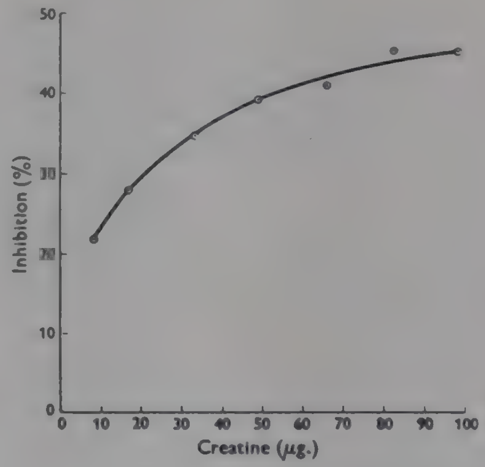

Fig. 3. Variation in the inhibition of the Barritt reaction in the presence of a constant ratio of cysteine ester : creatine of 0·6 : 1.

Application of the method to the estimation of creatine in tissue extracts

Well fed, adult, male rats of about 300 g. body weight were stunned by a blow on the head and the neck vessels severed. Samples of the various tissues were weighed in a mortar of the Potter & Elvehjem (1936) type, and homogenized with 5 ml. of 10 % (w/v) trichloroacetic acid (*TCA*). After centrifugation, the residue was re-extracted with 10 ml. of 5 % *TCA*. The combined supernatant fluids were made up to 25·0 ml. with water and filtered prior to the removal of samples for estimation.

Procedure. The creatine was determined in suitable samples of the *TCA* extracts both in the presence and absence of *CMBA*. To obtain some idea of the absolute amounts of reducing substances present, and hence the amount of *CMBA* necessary to combine with the SH present, samples of the *TCA* extracts were titrated with 0·01 N-iodine after the addition of 0·5 vol. N-HCl (to facilitate comparison with the previous experiments, the iodine titres are expressed as equivalents of cysteine ester). If it be assumed that the reducing substances are entirely thiol in character the addition of an equivalent amount of *CMBA* will ensure an excess of the reagent. The figures quoted in Table 5 are the means of at least two determinations on each sample.

Table 5. *Creatine content of rat tissues found in the presence and absence of CMBA*

Tissue	Cysteine ester equivalent (mg./100 g.)	Creatine (mg./100 g.)		Molar ratio cysteine ester equivalent : creatine	Increase with *CMBA* (%)
		CMBA absent	*CMBA* present		
Liver (1)	185	0	4·6	28·8	∞
(2)	244	0	3·8	45·7	∞
Spleen (1)	161	5·9	15·8	7·2	197
(2)	181	3·9	15·3	8·4	292
Kidney (1)	139	7·7	20·5	4·8	166
(2)	159	9·2	22·9	4·9	150
Lung (1)	124	6·0	13·0	6·8	117
(2)	149	5·8	11·8	9·0	104
Smooth muscle (small intestine) (1)	124	24·2	40·4	2·2	67
(2)	147	20·7	34·8	3·0	68
Testis (1)	172	248·0	295·0	0·4	16·5
(2)	178	260·0	299·0	0·4	15
Brain (1)	84	114·5	130·0	0·5	14
(2)	136	115·0	133·0	0·7	15
Cardiac muscle (ventricle) (1)	110	170·0	192·0	0·4	13
(2)	147	197·0	212·0	0·5	7
Skeletal muscle (1)	46	516·0	520·0	0·06	2
(2)	39	500·0	511·0	0·05	2

It will be noticed that in the case of kidney, spleen, lung and small intestine, the addition of *CMBA* produced a considerable increase in the amount of creatine estimated (Table 5). In the case of liver, the addition of *CMBA* now renders possible the determination of the creatine present, zero values being recorded in its absence. With the remaining tissues investigated, voluntary muscle, testis, brain and cardiac muscle, the increases are not as great, a maximum mean increase of only 15·7 % being present in the case of testis.

The magnitude of the increase in the amount of creatine measurable in the presence of *CMBA* will depend on the relative amounts of thiol and creatine. The method employed to obtain the cysteine ester equivalent, and thus the ratio of this to creatine, will lead to a high result. Nevertheless it is of some interest to note that there is a fair correlation between the ratios so obtained and the percentage increase in the creatine measured in the presence of *CMBA*. Thus small intestine, kidney, lung and spleen with ratios (obviously too high, cf. Fig. 1), of 2·6–7·5 : 1 show increases from 70 to 250 %, and the other tissues with ratios of 0·05–0·5 : 1 have increases of 2–16 %. In the case of liver tissue, a mean ratio of 37·2 : 1 will prevent the estimation of creatine in the absence of *CMBA* and thus the addition of this compound must lead to an infinite increase.

Although the figures for the creatine content of the various tissues examined are the maximum obtainable in the presence of *CMBA*, they do not necessarily represent the true creatine values because of the possible inhibition by substances other than the *R*-Hg-S*R'* compound formed and the excess *CMBA* present. This possibility was examined by attempting to recover 20 μg. of creatine which were added to samples before analysis.

Appropriate samples of the *TCA* extracts were taken in quadruplicate, and to each set of tubes 2·0 ml. of 5×10^{-2} M-*CMBA* (1426 μg.) were added. To two of the tubes in each series 20 μg. of creatine were added. α-Naphthol and diacetyl were added as before and the colour measured after 30 min. In view of the inhibition produced by the excess of *CMBA*, the amount of creatine was obtained by reference to a standard curve prepared by measuring the colour produced by known amounts of creatine in the presence of 1426 μg. of *CMBA*.

The recoveries of 20 μg. of added creatine (Table 6) indicate that this procedure will give values for the creatine contents of tissues which are reasonably close to those actually present.

Table 6. *Recovery of 20 μg. creatine added to trichloroacetic acid extracts of various rat tissues in the presence of CMBA*

Tissue	Creatine found (μg.) No addition	Creatine found (μg.) +20 μg. creatine	Recovery of added creatine (%)
Muscle	28·4	48·4	100
Testis	18·0	37·4	97
Lung	13·6	31·6	90
Brain	22·2	41·0	94
Cardiac muscle (ventricle)	20·2	41·0	104
Liver	18·2	36·2	90
Spleen	13·2	30·8	88
Kidney	43·4	60·8	87
Smooth muscle (small intestine)	41·3	59·9	93

As a matter of interest the behaviour of the α-naphthol-diacetyl method for the estimation of creatine in whole blood has also been studied (Table 7).

Table 7. *Creatine content of whole blood found in the presence and absence of CMBA*

(10·91 g. of rabbit blood + 10 ml. of 10% (w/v) trichloroacetic acid, proteins separated by centrifugation. The insoluble protein residue re-extracted with a further 10 ml. of 5% trichloroacetic acid, centrifuged and the supernatant fluids combined and made up to 25·0 ml. with water. Filtered. 2·0 ml. samples taken in all cases.)

Creatine in sample (μg.) *CMBA* absent	Creatine in sample (μg.) *CMBA* present	Creatine in sample (μg.) +20 μg. creatine, *CMBA* present	Increase in presence of *CMBA* (%)	Recovery (%)
14·6	18·6	36·2	27·4	88

Creatine, mg./100 g. blood = 2·4 (calculated from creatine measured in sample in the presence of *CMBA*).

Here again the recovery of added creatine may be carried out satisfactorily and is comparable in magnitude with that observed in tissue extracts. The influence of SH is again well illustrated by the 27% increase in the amount of creatine measured in the presence of added *CMBA*.

Some experiments have been carried out with urine from normal males, young children and pregnant females. These have been neither sufficiently numerous nor comprehensive to quote in detail, but some of the preliminary results are of interest. It has been found that some degree of inhibition is present in 1·0 ml. of urine as judged by the increased values in the presence of *CMBA*. These increases were variable and have not been correlated with other factors, but were of the order of 10%. 100% recoveries of added creatine were obtained by the addition of *CMBA*, and it therefore appears possible that this modification may prove useful for the estimation of creatine in urine.

DISCUSSION

It has been suggested by Eggleton *et al.* (1943) that ω-amino-acids may be responsible for the failure of the Barritt reaction to estimate creatine in *TCA*-liver extracts and in urine. They further state that extracts of most animal tissues, with the exception of liver, 'appear to contain insignificant amounts of interfering substances'. An examination of the ethanol-insoluble barium salts obtained from *TCA* extracts of liver led us to the conclusion that GSH was more likely to be the agent responsible for the inhibitory effect, and further that it is the SH group which is the determining factor.

The inhibitory influence of SH-containing compounds on the α-naphthol—diacetyl reaction for the estimation of creatine is very striking and depends on the molar ratio of SH : creatine. In the estimation of creatine in spleen, kidney, lung and intestine, the SH : creatine ratio is sufficient to produce errors ranging from 60 to 250%.

The inhibitory action of SH can be removed almost completely by the addition of *p*-chloromercuribenzoic acid, and the figures obtained in the presence of this compound suggest that the method, so modified, is now capable of giving creatine values which are close to the true values. An effect, similar to that obtained with this substance, could probably be gained by the addition of other organic mercurials and, in fact, by any substance reacting with SH groups to give a complex stable to alkali; but since *p*-chloromercuribenzoic acid behaved satisfactorily in our hands this has been used exclusively.

The results of experiments with pure SH compounds and SH-containing tissue extracts in the presence of *p*-chloromercuribenzoic acid strongly suggest that SH compounds are the only inhibitors of importance as far as the application of the Barritt reaction to tissue extracts is concerned. This conclusion is further strengthened by the fact that, in most instances, recoveries of known amounts of added creatine were 90% or greater. Although Eggleton *et al.* (1943) have observed a 50% reduction of the colour produced by 30 μg. of creatine in the presence of 4 mg. of glycine it seems improbable that such amounts of any amino-acid could be present in the usual samples taken for estimation of creatine in *TCA*-tissue extracts.

The only body fluids examined have been whole blood and urine, and in both cases the addition of *CMBA* appears to overcome the inhibitors which are present. The suggestion of Walpole (1911) that it is the NH_3 which interferes with the reaction in normal urine has not been confirmed.

It follows from the high degree of sensitivity of this reaction to the presence of sulphydryl compounds that use might be made of this fact for the detection, and possibly the estimation, of small amounts of SH and alkali-labile SS substances. Experiments are in progress to test this possibility.

As Barritt (1936) has pointed out, the reaction between diacetyl, α-naphthol and creatine to form a coloured product is also given by other guanido compounds. Eggleton *et al.* (1943) have stated that arginine, guanidine and glycocyamine produce about one ninth of the colour produced by equivalent amounts of creatine. All results quoted in this communication have been calculated on the assumption that creatine alone produces the colour measured. It is believed that the error introduced by such an assumption is small. However, in the application of the revised method, the possibility of increased amounts of other guanido compounds in pathological states must be borne in mind.

SUMMARY

1. The α-naphthol—diacetyl reaction for the estimation of creatine has been investigated.

2. The inhibition, which has been noted by other workers to occur when the estimation of the creatine content of trichloroacetic acid extracts of liver is attempted, has been shown to be due to the presence of sulphydryl compounds and, by inference, to the presence of glutathione.

3. The inhibition by sulphydryl compounds depends on the molar ratio of the sulphydryl compound to creatine, and is therefore most marked in the liver where the creatine content is low and the glutathione content high.

4. The ratio of sulphydryl compounds to creatine, in several tissues investigated, is sufficiently high to produce very gross errors in the estimation of creatine by this method. Exceptions are found in voluntary and cardiac muscle, brain and testis, but even here the method is subject to error.

5. The presence of disulphide compounds such as GSSG also produces an inhibition due, presumably, to the tendency of the disulphide linkage to split in alkaline solution.

6. The inhibition produced by sulphydryl compounds can be almost completely removed by the addition of *p*-chloromercuribenzoic acid; both this compound and that formed by its reaction with the sulphydryl compounds possess weak inhibitory powers.

7. The creatine content of various tissues, blood and urine has been determined both in the presence and in the absence of *p*-chloromercuribenzoic acid and an assessment made of the errors introduced by the omission of this compound.

8. The recoveries of creatine added to extracts of various tissues, blood and urine are mostly in excess of 90 %, thus indicating the general applicability of the modified method.

We wish to express our thanks to the Chief Scientist of the Ministry of Supply for gifts of methionine and benzyl mercaptan and to Messrs E. Facer and S. Moorbath for skilled technical assistance.

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1948

The Fermentation of Cellulose *in vitro* by Organisms from the Rumen of Sheep

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The suggestion that micro-organisms are responsible for the decomposition of cellulose in the alimentary canal (Wildt, 1874; Zuntz, 1879; Duclaux, 1882) arose from the proof (Henneberg & Stohmann, 1860–4) that it is, in fact, cellulose which disappears during the passage of crude fibre through the animal (Haubner, 1855). Tappeiner (1882, 1883, 1884, 1888), who was the first to investigate the fate of cellulose, found that paper pulp and cotton wool, when incubated under anaerobic conditions with the fluid contents of the rumen, dissolved slowly with the evolution of methane and carbon dioxide and the formation of fatty acids. He ascribed little nutritional value to the fatty acids, however, and suggested that the fermentation of cellulose was important only because it allowed the digestive enzymes access to the contents of the plant cells (Tappeiner, 1884). This view was not accepted (Henneberg & Stohmann, 1885); the fact that only traces of fatty acids appear in the excreta (Wilsing, 1885; von Knieriem, 1885) implies at least that they are absorbed and oxidized. There was already positive evidence of the nutritional value of cellulose (von Knieriem, 1885; Lehmann, 1889). Apart from the investigations of Markoff (1911, 1913) which added little to the information provided by Tappeiner (1884) this aspect of ruminant digestion was abandoned in a state of considerable confusion.

Tappeiner (1888) distilled the medium from somewhat prolonged *in vitro* fermentation of cellulose by rumen contents, and from the combining capacity and composition of the silver salts of the mixed volatile fatty acids in the distillate, claimed that acetic and butyric acids were the main products of fermentation. Quite apart from the possibility that his cultures had been qualitatively affected by the environment in which the fermentations were carried out, the chemical methods upon which the claim was based were not capable of distinguishing between mixtures of these two acids and any combination of the lower homologues. Examination of his data reveals no sound reason for the conclusion that only the 2-C and 4-C acids were among the products. On the occasion when he examined a series of fractions collected during progressive distillation Tappeiner (1884, p. 91) concluded, ‘Zweifelhaft bleibt das Vorkommen von Propionsäure’. His data clearly indicated the presence of considerable quantities of propionic acid.

Markoff's cultures also were probably subject to environmental alteration, and apparently he was unaware that the higher homologues distil first from a mixture of fatty acids in aqueous solution, and that an analytical technique based on distillation constants had been published by Duclaux (1874, 1895, 1900). He concluded without sound reason that the average molecular weight of the fatty-acid products of his fermentations of cellulose was the same as that of butyric acid (Markoff, 1913, p. 36). There was thus no real justification for the assumptions which led Honcamp (1909, 1927) to derive empirical equations for the degradation of cellulose in the alimentary canal.

Before the experiments described here were started* we were aware that considerable quantities of propionic acid appeared in the rumen during fermentation, but acetic acid usually predominated and sometimes accumulated in greater excess over the propionic acid than would be expected even if the pentosans of the fodder were fermented preferentially (Seillière, 1910) to acetic acid. This fact was difficult to reconcile with our observations on the apparent overall usefulness to the animal of the products of rumen fermentation. It appeared from the work of Lusk and his colleagues (Ringer & Lusk, 1910; Lusk, 1921; Milhorat & Deuel, 1926–7) that, in the dog at least, acetic acid was rapidly and wastefully oxidized without entering the carbohydrate cycle; and it was obvious from energy transactions that the ruminant derives more useful energy from its fodder than would be implied if the greater proportion of the carbohydrates were converted to acetic acid and subsequently disposed of without entering into the productive channels of metabolism.

Although these *in vitro* fermentations were conducted under conditions as similar to those obtaining in the rumen as were technically feasible, a considerable preponderance of propionic acid accumulated. This finding was in more reasonable agreement with the results of some calorimeter experiments on sheep carried out in this laboratory, which we hope to publish shortly, but the (acetic)/(propionic) ratio was so different from that found in the rumen contents as to lead to a suspicion that the community of micro-organisms responsible for the fermentations *in vitro* had been so altered that the course of degradation had been changed materially from that which normally prevails in the paunch.

The confirmation by Barcroft and his colleagues of Trautmann's (1933) observation that absorption may take place directly through the rumen wall (Barcroft, McAnally & Phillipson, 1944*a*), and their convincing demonstration that considerable quantities of fatty acids find their way into the blood stream draining the rumen (Barcroft *et al.* 1944 *b*, *c*), provided the clue which led to a more complete understanding of ruminant digestion and to a better appreciation of the energy transactions involved in the utilization of the fermentation products. Further experiments have shown that when the contents of the rumen are acid, as they normally are during active digestion, propionic acid is absorbed preferentially to acetic acid (Danielli, Hitchcock, Marshall & Phillipson, 1945; Gray, 1947). The relative rates of diffusion through the rumen wall are such as may normally reverse the ratio of acetic/propionic acids in the contents of the rumen. Conclusions based only on analyses of rumen contents may thus be misleading. The necessity for further knowledge of the products of *in vitro* fermentations conducted in an environment similar to that of the rumen is obvious.

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* The experiments were carried out in part at the Biochemical Laboratory, Cambridge, during 1937 when the author was the guest of the late Sir Frederick Gowland Hopkins, and in part at the author's laboratory, Adelaide, in 1938.

The environment in the rumen

The environment in the rumen is the main factor which regulates the community of micro-organisms responsible for carbohydrate degradation. Outstanding features of this environment are:

(*a*) *Salivary secretion.* A relatively large quantity of dilute alkaline saliva is secreted continuously by the parotid glands.* Besides its buffering capacity and its ability to maintain a high CO_2 tension in the medium where the fermentation proceeds, the parotid saliva carries to the micro-organisms a supply of phosphate and simple nitrogenous compounds, which is in very considerable excess of the quantity usually available from the fodder.

(*b*) *The reaction of the rumen contents.* When in a quiescent state, 24 hr. after ingestion of food, the rumen contents are about neutral. In samples from twelve animals taken at slaughter and collected without loss of CO_2, the reaction at 40° ranged between pH 6·40 and 7·14, with a mean of pH 6·8. The fatty acids produced during fermentation are in part neutralized by the saliva, and in part removed either by passage to lower levels of the alimentary canal or by direct diffusion through the rumen wall. When fermentation is most vigorous, during the period between 4 and 8 hr. after feeding, acid production exceeds these modifying influences; the mass then becomes distinctly acid, sometimes less than pH 5.

(*c*) *The oxidation-reduction condition of the liquid contents and the gas tension of the atmosphere above.* The partial pressure of oxygen is usually less than 8 % in the atmosphere above the liquid contents of the quiescent rumen 24 hr. after feeding. This is augmented temporarily by the oxygen in the air swallowed with the food. It is diminished abruptly to very low levels (cf. Washburn & Brody, 1937), mainly through displacement by the CO_2 and the methane evolved when active fermentation begins soon after feeding. The liquid contents of the rumen, however, maintain a low oxidation-reduction potential and, even after the animal has fasted 3 days, the conditions are such as to allow some methane production to proceed. Normally there is a high partial pressure of CO_2 in the rumen gas at all times.

Experimental imitation of the milieu

The low partial pressure of oxygen and the high CO_2 tension, the supply of phosphate and inorganic nitrogen from the saliva, the buffering capacity of the saliva, the temperature of approximately 40°, and the residual contents of the rumen, which retain a massive inoculum of a community of micro-organisms already selected by the *milieu*, are apparently the main factors which determine the course of fermentation in the normal rumen. When subjected to these conditions, the complex carbohydrates and simple sugars are fermented very rapidly. Some observations (unpublished) on the rate of disappearance of cellulose from different levels of the intestinal tract of sheep indicated that a digestion mass of approximately 5 l. in the rumen of a sheep fermented in 8 hr. from 400 to 600 g. of mixed carbohydrates, at least half of which was cellulose, and that the rate of dissimilation at the peak of activity exceeded considerably the average over this period. The rapid rate of fermentation and of absorption of the products was reflected in the energy metabolism of the sheep: after feeding with 1 kg. of hay the metabolic rate increased within an hour to a level approximately 25 % above that previous to feeding, remained there for 6–7 hr., and then receded during the ensuing 2 hr. to the lower level which was maintained until the animal was fed again 24 hr. later.

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* Colin (1886) estimated that approximately 60 l. of parotid saliva are secreted each day by the ox. Scheunert & Trautmann (1921) concluded that the sheep secretes approximately 4 l. in 24 hr., which is probably a low estimate. In an investigation preliminary to these *in vitro*-fermentation experiments, the author established fistulae in the left parotid ducts of three mature ewes and, when the secretion was passed back into the mouth at frequent intervals, collected up to 3·5 l. of saliva in 24 hr., which would imply the secretion of approximately 7 l. each day under the particular feeding conditions of the experimental animals. They were fed at noon each day with 1·2 kg. of a mixture of chaffed cereal and lucerne hays, and consumed the whole of it within 2 hr. (cf. Marston, 1939). In these studies there was evidence of reflex stimulation by the acidity of the rumen contents, and so a reasonable estimate was possible only if the secretion was collected during the whole cycle of 24 hr. Chemical analyses of the saliva confirmed Scheunert & Trautmann's (1921) estimate of the bicarbonate concentration (equivalent to 0·1–0·2 N-$NaHCO_3$) and indicated that both the phosphate and the urea, which became concentrated in the secretion, reflected changes in the inorganic phosphate and urea concentrations of the blood. Watson (1933), in this laboratory, had established previously that the concentration of phosphate in the blood was related to that of the mixed saliva collected on sponges inserted into the mouth.

EXPERIMENTAL

Apparatus. The vessel in which the fermentations were carried out was a 3·5 l. glass pot, hermetically sealed with a metal lid clamped down on a rubber gasket (Fig. 1). A stirrer attached to a geared variable-speed motor which could at will be run very slowly, passed through a mercury-sealed bearing in the lid. Six ports, tapered to fit standard rubber stoppers, through which other pieces of apparatus could be introduced, were attached to the lid. All metal parts within the pot were coated with paraffin wax. The subsidiary apparatus (Fig. 2) consisted of: an inlet and an outlet tube for gas, *A*, one of which was constructed to take the delivery point of a burette through which the standard alkali could be introduced; a standardized glass electrode for pH measurements, *B*; a bright platinum electrode, *C*, for oxidation-reduction potential measurement; a saturated calomel half cell, *D*, to provide a reference potential; a closed pipette, *E*, into which a sample of the liquid contents of the pot could be drawn up around a second glass electrode which was used on occasions to check the accuracy of the glass electrode, *B*; and a toluene-mercury thermoregulator which, through a relay, operated the heating elements of the water bath in which the pot was immersed. The transfer of heat through the pot was not sufficiently rapid to ensure very precise temperature regulation: the contents varied ±1·5° about the 40° setting.

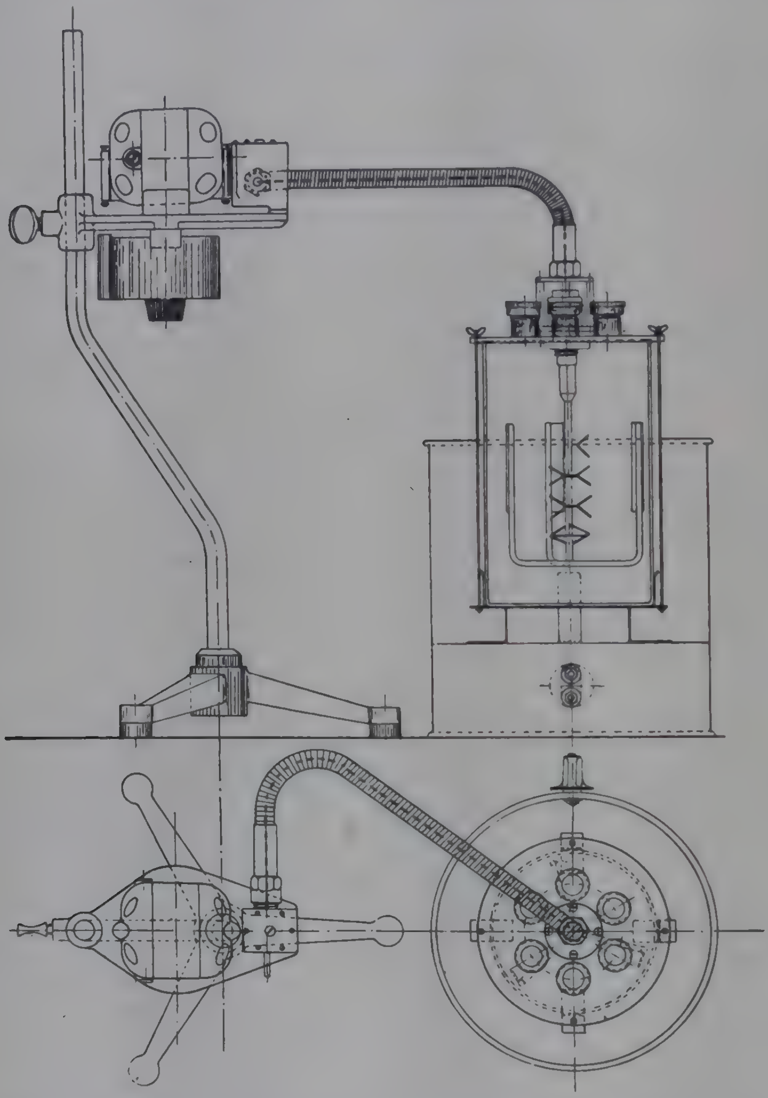

Fig. 1. Fermentation apparatus consisting of a 3·5 l. closed jar with mercury-sealed stirrer and six ports through which subsidiary apparatus was introduced.

The ingoing stream of N_2 was purified by passing over soda-asbestos and then over freshly reduced Cu maintained at approximately 600° in a silica-tube furnace. The outgoing stream, after dilution with approximately twice its volume of CO_2-free O_2, was dried by passing through H_2SO_4 and then over anhydrous $Mg(ClO_4)_2$ (Dehydrite) and the CO_2 absorbed with soda-asbestos (Ascarite) in two vessels placed in sequence. These were large enough to deal efficiently with the considerable volume of CO_2 evolved as a fermentation product, and from the acid decomposition of the Na_2CO_3 which was introduced during the digestion. Guard tubes containing anhydrous $Mg(ClO_4)_2$ were introduced into the chain at each point immediately after the CO_2 absorbers. The dry, CO_2-free gas was then led through the combustion chain which is described in a later section.

Substrate, culture medium and inoculum. The cellulose used in Exps. 1 and 2 was a specimen provided by Dr David Bell and described by him as 'practically pure α-cellulose from birch wood'; in Exps. 3 and 4 it was prepared from filter paper (Munktell 1 F) which had been suspended in 17·5 % (w/v) NaOH for 1 hr., then washed with dilute HCl and subsequently with water until free from chlorides. It was then dried. Both samples were reduced to fine powder (100 mesh). In this state the cellulose was more tractable during the fermentation.

The salts in the inorganic medium were the usual 'analytical reagent' grade which, being by no means free from heavy metals, would probably have carried with them sufficient of these constituents to meet the requirements of the micro-organisms. However, to ensure that the course of digestion would not be influenced by the want of these minor elements which are present always in the normal fodder of ruminants, Fe, Cu, Zn and Co were added to the medium as solutions of the chlorides in sufficient quantity to provide 2 mg. Fe, 1 mg. Cu, 2 mg. Zn, and 0·5 mg. Co/l. of the inorganic medium.

For each digestion, approximately 50 g. (*vac.* dry wt. 100°) of the finely divided cellulose was weighed and suspended in 2·5 l. of a solution containing the following concentrations of inorganic constituents: 0·02 M-$(NH_4)_2SO_4$, 0·02 M-KH_2PO_4, 0·01 M-$MgSO_4$, 0·005 M-$CaCl_2$, together with the traces of heavy metals mentioned above.

The micro-organisms which were used as an inoculum were separated mechanically from the rumen contents of sheep which for some days had been fed on chaffed cereal hay. At slaughter, the paunch was tied off and the contents (3–5 l.) removed and strained through a sieve of fine gossamer while being vigorously stirred; this and all subsequent operations were conducted under an atmosphere of CO_2. The liquid portion was freed further from gross contaminating particles by gentle centrifuging. The micro-organisms in the supernatant liquid were then separated in a Sharples centrifuge. The residual sludge was removed from the cylinder, emulsified in 0·2 M-phosphate buffer, pH 5·5, and made up to 50 ml. The CO_2 was swept out with pure N_2. Samples for cellulose and carbon determinations were withdrawn and the remainder (which contained between 5 and 8 g. of wet sludge) was transferred without delay to the digestion vessel, already prepared to receive it. The material consisted largely of bacteria, the main contaminants being small fragments of lignin, lignocellulose and some of the micro-fauna which normally inhabit the rumen.

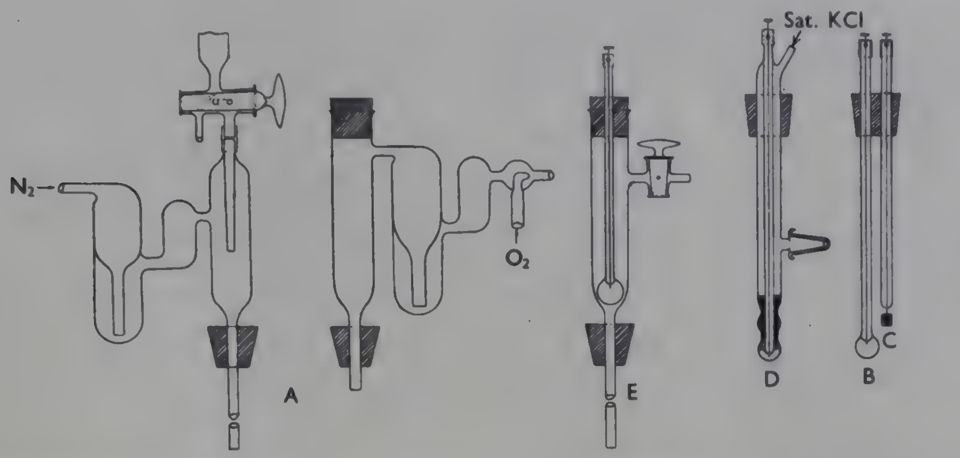

Fig. 2. Subsidiary apparatus introduced through ports in fermentation apparatus: inlet and outlet tubes for gas, etc., and glass, calomel and platinum electrodes. The thermoregulator introduced through the sixth port is not shown.

Procedure of fermentation. After introducing the cellulose, the vessel was closed, the subsidiary apparatus made ready and the contents raised to 40°. The inoculum was run in through one of the ports. The air was swept out with a rapid stream of pure N_2 passed through the inlet tube beneath the surface while the mash was being stirred rapidly. The inlet tube was then withdrawn above the surface, the flow of gas reduced to about 300 ml./hr. and the efferent stream, after dilution with O_2, diverted to the absorption and combustion chain. Standardized 0·5 M-Na_2CO_3 was added drop by drop from the burette, with gentle stirring, until the reaction of the contents was pH 6·8. Stirring was then stopped. Active digestion usually began within an hour and became increasingly rapid. The rate was judged by the change in reaction of the contents as indicated by the glass electrode. The reaction was adjusted at frequent intervals (10–20 min.) by introducing standard Na_2CO_3 from the burette; it was kept by this means between pH 6·8 and 5·5 throughout the course of each fermentation. The stirrer was used only when adjusting the reaction, and then at a rate sufficient merely to impart a gentle swirl to the medium. Rapid stirring was found to reduce the rate of fermentation.

In this way the *milieu* of the rumen was imitated. The conditions were at least very similar to those which prevail normally. The inoculum was relatively massive, and there was a minimum of selection of micro-organisms by the mechanical processes employed to separate them from the normal rumen contents; the reaction of the medium was controlled; the conditions were anaerobic and the CO_2 tension high; and the duration of the fermentation was relatively short. It might reasonably be expected that the course of dissimilation of the cellulose under these conditions would be close to, if not identical with, that taken during normal digestion in the paunch of ruminants.

The results of four successful digestions are reported here.

The oxidation-reduction (O/R) potential of the medium during fermentation. During active digestion the potential set up on the indifferent (Pt) electrode immersed in the medium indicated an E_h at pH 7·0 poised in the vicinity of −0·380 V. The observed potentials fluctuated, sometimes rapidly, over a relatively wide range (approximately ±30 mV.). The system responsible seemed too delicately balanced to suggest even an approach to a state of true equilibrium; the more negative potentials suggested rather the production of evanescent, highly reducing intermediates, possibly of hydrogen itself. Active fermentation of cellulose was invariably associated with such low potentials; from observations, however, it was not possible to conclude whether they represented an essential condition for the function of the organisms or whether they were merely the result of the formation of cellulose dissimilation products.

RESULTS

The products of the fermentations *in vitro*

Gaseous products: CO_2 and CH_4. Throughout the course of each experimental digestion the atmosphere above the fermenting mass was swept with pure N_2, continuously, but sufficiently slowly to ensure that the CO_2 tension was not reduced to low levels. The efferent stream, after it had been diluted with pure O_2 and freed quantitatively from H_2O and CO_2, was led through a silica tube packed with CuO and maintained at approximately 700°, and thence through anhydrous $Mg(ClO_4)_2$ and soda-asbestos absorbers to collect the H_2O and CO_2 arising from the combustion. At the completion of a digestion period the introduction of alkali was stopped, the mass was allowed to become acid (pH 5·8–6·0) and then 5 ml. H_3PO_4 (sp.gr. 1·6) were delivered through the inlet port. The N_2 delivery tube was pushed well down beneath the surface, the stream of gas increased, and the mass agitated vigorously with the mechanical stirrer. This procedure was not prolonged further than would ensure removal of the fermentation gases without washing out any appreciable quantity of the more volatile products of digestion.

Methane was the only combustible gas evolved under these conditions of fermentation. Within the very small error of the estimation there was no evidence that the gas evolved contained any free hydrogen: the ratios of CO_2 and H_2O arising after combustion invariably indicated a carbon/hydrogen ratio of 1 : 4 atoms.

The formation of methane from hydrogen has been recognized since Söhngen (1905) demonstrated the reduction of CO_2 by H_2 with enrichment cultures from soil. Its formation as a product of the CO_2 oxidation of ethanol by *Methanobacterium omelianskii* (Barker, 1936*a*) or of simple fatty acids by *Methanococcus mazei* and by *Methanobacterium söhngenii* (cf. Barker, 1936*b*) are well established reactions. Its production from any compound containing a carbon chain, by an organism isolated by Stephenson & Stickland (1933), reveals further the variety of possible sources of methane in anaerobic fermentations with mixed cultures. The fact that free hydrogen is evolved during the dissimilation of cellulose by 'enrichment' and by 'pure' cultures, or by mixed cultures under conditions which favour thermophilic organisms but in which methane producers could not function, suggests that the bulk of the methane evolved from the rumen may be formed from hydrogen during primary fermentation of complex polysaccharides. Decomposition of other products would be a subsidiary source of methane. Both types of reaction almost certainly occur.

There is thus no reason to expect an inflexible relationship between the amount of methane produced, the amount of substrate fermented, and the CO_2 evolved in the process.

The ratios $(CO_2)/(CH_4)$ in the four fermentations under discussion varied between 3·0 and 3·8 (see Table 2). As the estimation of these constituents was precise, this variation may be attributed to differences in the course of the activity of the mixed cultures. The changes in the ratios do not correlate simply with similar changes in the ratios of the volatile fatty acids, nor with the degree of exhaustion of the substrate. The differences might thus have been due in these experiments to changes in the nature of the original inoculum. The partial pressure of CO_2 above the fermenting mass was not rigidly controlled and may at times have deviated from that which normally prevails in the rumen during active digestion. The fact that free hydrogen was not evolved indicates, however, that at least the CO_2 oxidation of hydrogen was not at any time limited by the CO_2 tension. Between 6·2 and 8·1 % of the combustible energy of the cellulose digested was contained in the methane evolved.

The main products: fatty acids. The major part of the undissolved cellulose was collected on a sintered glass funnel. The organisms and fine particles of cellulose which passed the filter were then spun off in a Sharples centrifuge and the residue washed. The insoluble fractions were combined and the cellulose content determined by the method of Norman & Jenkins (1933).

The clarified filtrate and washings were adjusted with NaOH to pH 8·0 and evaporated to about 250 ml. in a still, to which was attached an efficient condenser and a close-fitting receiver immersed in a freezing mixture. Steam was then admitted to the flask and the distillation continued until an additional litre of distillate had collected in the cold receiver. The distillate (D_1) was preserved for the estimation of volatile alcohols, aldehydes, ketones, etc. The residue in the flask was concentrated to approximately 250 ml., acidified with H_3PO_4 to pH 3·0 and then steam distilled without increasing its volume, until approximately 5 l. of distillate (D_2) had collected. The residue (R_1) was preserved for the estimation of succinic acid and the semi-volatile lactic and pyruvic acids. The distillate (D_2) was returned to the still, rendered alkaline, and the volume reduced again to approximately 250 ml. The distillate (D_3) from this, after applying qualitative tests for alcohols, ketones, etc. was discarded. The residue in the flask was acidified again to pH 3·0, and steam distilled until 5 l. of distillate (D_4) had been collected. The residue from this (R_2) was combined with (R_1) and treated as described in the section on intermediates. The distillate (D_4) was exactly neutralized with a known quantity of $Ba(OH)_2$, computed from careful titration of a sample, and then cautiously reduced to dryness and dried finally in a vacuum desiccator. From the weight of the Ba salts and the Ba content the mean combining capacity of the mixed salts was computed. This provided a useful means of evaluating the overall accuracy of the subsequent analyses.

The Ba salts were then taken up in water, decomposed with the calculated quantity of H_2SO_4, and the mixed fatty acids extracted with pure ether in a continuous extraction apparatus. After removal of the ether they were fractionated in an apparatus designed specially for quantitative separation of mixtures of this sort. The column employed was an unpacked spiral glass tube, approximately 10 m. in length, sealed together with a totally enclosed copper reflector into a glass vacuum jacket, the whole being encircled externally with an electrically heated casing. This latter and the external insulation allowed precise adjustment of the thermal gradient. The rate of entry of vapour into the column was determined by a thermoregulator situated at its head, and means were provided for controlling the quantity of liquid taken off. When the apparatus was adjusted critically a sharp separation of the constituents was ensured with a minimum of retention in the fractionating column. The efficiency of the column was equivalent to 24 theoretical plates.

The following fractions were collected: F_1, up to 101°, which contained water and formic acid; F_2, from 101 to 119°, which contained the acetic acid; F_3, from 119 to 142°, which contained the propionic acid; F_4, from 142 to 164°, which contained butyric acid. Transition between the fractions was abrupt. Formic acid was determined in F_1 by reduction of HgO (Osburn, Wood & Werkman, 1933) and butyric acid in F_4 and in the residues by oxidation to acetaldehyde (Klinc, 1934). The composition of fractions F_3 and F_4 was established further by the refractive index and by the combining capacity and the composition of the anhydrous barium salts. The presence of higher homologues in the residuum was doubtful: if they did occur they were in very small quantities.

The results from complete analyses of the products of four digestions are set out in Table 1. The minor products were not determined in digest 1, but in subsequent experiments these were sought for and estimated quantitatively. They were unimportant in the overall balance sheets of the transactions involved, and the estimations added but little to the understanding of the intermediary stages of the dissimilation.

Table 1. *The products of fermentation of cellulose*

(mmol. = millimols/'mol' (162 g.) of cellulose digested.)

Digest	Formic acid	Acetic acid	Propionic acid	Butyric acid	Methane	CO_2	Cellulose digested (g.)	Duration o digestion (hr.)
1	0·08 g. 12 mmol.	5·3 g. 600 mmol.	11·6 g. 1060 mmol.	0·27 g. 21 mmol.	0·6 g. 250 mmol.	5·4 g. 830 mmol.	24	26
2	0·17 g. 34 mmol.	4·0 g. 610 mmol.	8·5 g. 1050 mmol.	0·3 g. 31 mmol.	0·46 g. 260 mmol.	3·9 g. 810 mmol.	17·8	24
3	0·11 g. 11 mmol.	7·8 g. 620 mmol.	14·4 g. 925 mmol.	1·68 g. 91 mmol.	0·66 g. 195 mmol.	6·9 g. 750 mmol.	34	36
4	0·21 g. 44 mmol.	3·1 g. 500 mmol.	6·4 g. 840 mmol.	1·2 g. 130 mmol.	0·39 g. 236 mmol.	3·2 g. 710 mmol.	16·7	48

The most striking of the analytical findings was the proportion of the main products, propionic acid and acetic acid, and their relationship to the cellulose digested. This relative constancy in the four digests could hardly be fortuitous: it indicates a strong tendency for the main course of dissimilation to proceed through similar if not identical channels. Either the populations of micro-organisms which constituted the inocula were of extraordinarily similar composition, or the selective forces of the environment brought about a relatively stable associative growth. It is probable that the latter were mainly responsible. The inocula were separated from the rumen contents of sheep all of which had been fed similarly for a considerable period prior to slaughter. Each population of micro-organisms thus had ample opportunity to become stabilized.

Table 2. *Molecular ratios of main constituents*

Digest	(Propionic)/(Acetic)	$(CO_2)/(CH_4)$
1	1·8	3·3
2	1·7	3·1
3	1·5	3·8
4	1·7	3·0

The ratios of propionic acid and acetic acid produced in these digests (Table 2) were so similar to those found by Van Niel (1928) to result from the fermentation of lactic acid by certain strains of *Bact. acidii propionici*, as to suggest that organisms of the genus *Propionibacterium* were concerned in their production. Formic acid and butyric acid were present in all digests. The amounts were small and their proportions much more variable. It would appear that these are the products of subsidiary and relatively unstable channels of dissimilation.

Elsden (1945), employing partition chromatographic means (Martin & Synge, 1941; Smith, 1942; Ramsey & Patterson, 1945; Elsden, 1946) for the separation of the fatty acids, found in the rumen contents a preponderance of acetic over propionic acid, but he recognized the fact that propionic acid predominated over acetic acid when carbohydrates were fermented under anaerobic conditions in inorganic media inoculated with liquor withdrawn from the paunch. The findings from the *in vitro* fermentations under discussion here are in substantial agreement except for the wide variability of the acetic/propionic ratios found by Elsden. There is, however, no real reason to conclude that the normal course of carbohydrate dissimilation in the rumen varies to the extent implied by Elsden's observations. He made little serious attempt to duplicate the *milieu* of the rumen; the inocula employed were small and the duration of the fermentations prolonged. The possibility that the course of the dissimilation might be altered under such circumstances was greater than when the conditions of the rumen were imitated and the environment controlled throughout.

Intermediate products. In mixed cultures of this sort, the metabolic end products of the organisms responsible for primary fission of the cellulose would be exposed to further dissimilation. In addition, each soluble intermediate would be subject to the potential competitive action of other organisms, always attendant and ready with a wide diversity of enzyme systems to take advantage of suitable substrates. Intermediary products under such circumstances would be more than usually evanescent and the end products themselves to some degree transient, for the extent of accumulation would be a measure of relative, rather than of complete, stability in this environment.

An exhaustive study of the intermediate compounds may thus not greatly illuminate the processes involved in these mixed fermentations. A search was made, however, for products of subsidiary channels of dissimilation, which might accumulate in sufficient concentration to be of some nutritional importance to the ruminant. The proportions of propionic and acetic acids in digest 1 suggested that the main course of the dissimilation might proceed through lactic acid (see above). For this reason lactic acid was estimated in digests 2, 3 and 4. The following data from digest 2 are typical of the findings.

Reducing sugars were absent. The distillate (D_1) contained 13 mg. acetaldehyde. Residues (R_1) and (R_2) were combined, reduced in volume, then acidified and exhausted with ether by continuous extraction. The small quantity of extractive, after freeing from ether, was taken up in water and the succinic acid, the aldehydes and ketones, and the semi-volatile lactic and pyruvic acids were estimated. Oxidation of a sample by a modification (Friedemann, Cotonio & Shaffer, 1927) of the von Fürth & Charnass (1910) procedure produced a quantity of acetaldehyde which would imply the presence of approximately 30 mg. lactic acid in the whole digest. The total sulphite-combining capacity of the extractive was of an order which would correspond to less than the equivalent of 6 mg. pyruvic acid, and this would be the upper limit of concentration. As more than 70 % of this was destroyed on heating with alkali the concentration of pyruvic acid must have been very small. The quantity was insufficient to allow it to be isolated as the 2:5-dinitrophenylosazone. About 7 mg. succinic acid were identified and about half this amount was isolated. A small quantity of an unidentified ketone or aldehyde was present.

It would appear that lactic acid rather than pyruvic acid tended to accumulate in these digests. This, however, is no proof that lactic acid was a more important intermediate than pyruvic acid in the dissimilation: the reverse might well be true. The prevention of the reduction of lactate by the addition of fluoride would have provided useful evidence on this point, but at the time when the experiments were conducted neither Barker & Lipmann (1944) nor Chaix-Audemard (1940) had published their interesting studies. Attempts were made, which however proved abortive, to accumulate intermediate complexes in the digest by applying Neuberg's *Abfangsverfahren*. Sulphite, when added to an actively fermenting culture apparently acted as a hydrogen acceptor, and altered the course of dissimilation. Hydrogen sulphide was evolved; nitrogenous compounds, which were either introduced in the original inoculum or had been elaborated since, underwent putrefactive changes; in contrast to the light colour and faint sweet odour of the contents of the normal rumen, or of the *in vitro* fermentations under discussion, the mass darkened and stank of indole and mercaptans. Intermediates accumulated by this means would have been of doubtful significance, and so the task of isolating them was set aside.

The minor products identified were not in sufficient quantity to be a nutritionally important energy source for the animal, and they were too small to be considered in the computations of the energy and carbon balance sheets.

The carbon and combustible energy balance sheets

In the four digests that were analyzed in detail, between 84 and 93 % of the carbon of the cellulose transformed was recovered in the main products of digestion (see Table 3). There was no evidence which would suggest that either the intermediary complexes or the products from subsidiary channels of dissimilation ever accumulated to an extent which would influence materially the carbon balance sheets. The losses incurred in the chemical isolations were not great, and the small experimental errors entailed in the estimation of the relative proportions of the fatty acids would have comparatively little influence on the assessment of the overall carbon content of these main products. It is not unreasonable, then, to conclude that by far the greater part of the carbon deficit could be accounted for by the carbon bound in the cells of the micro-organisms during their proliferation and growth.

An attempt was made to assess this directly. The main bulk of the undissolved cellulose of digest 3 was separated from the finer particles and micro-organisms by shaking vigorously and straining through fine gossamer. The gross residue was washed in this way several times. The washings were passed through the supercentrifuge after the original filtrate. Cellulose was estimated in the residues which did not pass the sieve and in the sludge from the supercentrifuge by the method of Norman & Jenkins (1933), and the total carbon in the latter was determined by the wet-digestion method of Backlin (1930). The difference between the total carbon content of these residues and the carbon of the cellulose in them was taken as carbon bound in micro-organisms, corrections being applied for the carbon introduced in the original inoculum. Approximately 75 % of the carbon deficit in the soluble products was accounted for in this way. It was at best an approximate estimate. Sampling difficulties were encountered, and only a partial recovery of micro-organisms was realized.

The proportion of the carbon of the digested cellulose which was present in the fermentation products was greater when the period of digestion was not prolonged. This suggests that as digestion proceeded, conditions became more favourable for the multiplication of organisms other than those which brought about the primary cleavage of the cellulose; the extent of proliferation of these latter might be expected to bear a more direct relationship to the amount of cellulose digested while this substrate was present in large excess.

The carbon and combustible energy-balance sheets (Tables 3 and 4) indicate that the cellulose fermentation products contained 6 % less combustible energy than that of the cellulose fermented. This deficit is an approximate estimate of the free

Table 3. *Carbon contents of the constituents of four digests analyzed in detail*

Digest	Formic acid carbon (g.)	Acetic acid carbon (g.)	Propionic acid carbon (g.)	Butyric acid carbon (g.)	Methane carbon (g.)	Carbon dioxide carbon (g.)	Cellulose digested carbon (g.)	Carbon recovered in products (%)
1	0·02	2·1	5·6	0·15	0·45	1·5	10·7	92
2	0·04	1·6	4·1	0·16	0·34	1·1	7·9	93
3	0·03	3·1	7·0	0·91	0·49	1·9	15·1	89
4	0·06	1·25	3·1	0·65	0·29	0·85	7·4	84

Table 4. *Combustible energy contents of the constituents of four digests analyzed in detail*

Digest	Formic acid (kg.cal.)	Acetic acid (kg.cal.)	Propionic acid (kg.cal.)	Butyric acid (kg.cal.)	Methane (kg.cal.)	Carbon dioxide (kg.cal.)	Cellulose digested (kg.cal.)	% of kg.cal. in products
1	0·1	18·5	57·5	1·6	7·9	—	100	86
2	0·2	14	42	1·8	6·0	—	74	87
3	0·1	27	71	10	8·7	—	140	83
4	0·3	10·8	32	7·1	5·1	—	70	79

energy in the conversion of cellulose to these products, as entropy changes in transformations of this sort are sufficiently small to be within the experimental errors entailed in the chemical estimations on which the computations were based. This energy would be available for the metabolic processes of the micro-organisms. As the relationships between the carbon and combustible-energy contents of cellulose and of the organisms which proliferate during these fermentations are approximately the same,* the amount of energy dissipated as heat in the exothermic metabolic processes of the micro-organisms would be practically identical with the free energy of the dissimilation. The energy balance sheet of the fermentation would thus be as set out in Table 5.

Table 5. *The energy balance sheet of the dissimilation*

	Combustible energy of cellulose fermented		
Digest	In soluble products and methane (%)	In micro-organisms (%)	Dissipated as heat (%)
1	86	8	6
2	87	7	6
3	83	11	6
4	79	16	5

The fact that the temperature of the rumen contents during active fermentation of hay is above body temperature was pointed out by Krzywanek (1929).

DISCUSSION

Rumen fermentation and the energy metabolism of the ruminant

The associative growth of organisms in the rumen

It seems likely that the course of dissimilation of cellulose in the rumen is determined primarily by the *milieu*, for it is highly probable that any of the vast variety of micro-organisms which inhabit the soil might find their way into the paunch. Those able to flourish anaerobically on the intermediary and end products of the primary fission of complex carbohydrates would form, with the organisms responsible for this first cleavage, a more or less stable population whose composition would be determined by the mutually beneficial relationships between the strains able to take their place in this community.

It might be expected that under the influence of the selective forces of the environment, the process of dissimilation by an associative growth of this sort would tend strongly to assume a pattern, which in many respects would be analogous to the orderly series of dehydrogenations which take place when the simpler carbohydrates are degraded under aerobic conditions in the animal organism.

Viewed in this light, it would not be remarkable if the nature and proportions of the products of fermentation in the rumen were similar, irrespective of the variations in composition of the substrate which obtain under natural feeding conditions. The simple fatty acids represent a relatively refractory stage of carbohydrate dissimilation in the environment of the paunch and, for this reason, accumulate. They are removed from the rumen too rapidly for secondary changes to assume any but an unimportant role. If, however, the *in vitro* digests are left exhausted of their substrate, further anaerobic degradation of these temporary 'end products' takes place. The final stage would probably be the complete conversion to carbon dioxide and methane found by Hoppe-Seyler (1886) when cellulose was fermented for 4 years with cultures from mud.

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* The combustible energy in bacteria of this type is close to 9·2 kg.cal./g. carbon, the carbon content being about 50 % of their dry weight and the combustible energy between 4·6 and 4·7 kg.cal./g. dry weight (Rubner, 1904). In cellulose it is 9·4 kg.cal./g. carbon, the carbon content being 44·4 % and the combustible energy 4·18 kg.cal./g. The former would thus be approximately 98 % of the latter.

The influence of starch and other supplements on the energy transactions in the rumen

If diets containing a high proportion of starch are fed, the rate of proliferation of the microflora in the rumen is limited usually by the nitrogen supply, for, when additional nitrogen is provided as ammonium salts or as simple amides, a large proportion of it is converted to protein through the rapid proliferation of micro-organisms. A considerable part of the combustible energy of the starch may, under these circumstances, escape in part the losses inevitably associated with fermentative degradation. The thermodynamic costs entailed in the production of these organisms are not known, and so the overall effects, which microbial syntheses in the presence of starch have on the supply of energy to the animal, cannot at present be assessed.

When the substrate is composed essentially of the cellulose, cellulosans and polyuronides, etc. of the natural fodder of ruminants, proliferation of the microflora is not influenced so materially by the nitrogen supply, and supplements of simple nitrogenous compounds are utilized poorly if at all by this population. The combustible energy in the bacteria, which pass from the rumen of sheep on this fodder, may be considerably less than the amount estimated from the *in vitro* fermentations discussed above, for *in vivo* the products of fermentation are removed from the rumen and so proliferation at their expense is minimized. In the overall energy economy of an animal on its natural fodder this course in the energy transactions of rumen digestion, although an important adjunct, is a subsidiary one; there is little doubt that the fatty acids arising from the dissimilation of carbohydrates in the paunch provide for the ruminant its main source of energy.

The usefulness of the fermentation products as sources of energy to the animal

The high degree of efficiency, with which the ruminant is able to derive energy from the more complex carbohydrates in its fodder, is offset by the losses associated with the fermentation of starch and of the simpler sugars. In other animals these latter suffer no appreciable loss of combustible energy when they are hydrolyzed by the enzymes of the alimentary canal. They are absorbed as simple sugars before the ingesta pass to the caecum, where micro-organisms ferment in part the more refractory carbohydrate complexes which are unchanged by the digestive enzymes.

The energy represented by the heat dissipated in the metabolic processes of the micro-organisms responsible for the dissimilation in the rumen would be of no benefit to an animal in a warm environment; under cold conditions, however, when the heat radiated by the animal exceeds that evolved by the normal processes of its metabolism, this fraction would spare its equivalent of the combustible energy of nutrients which otherwise would be oxidized in order to maintain body temperature. The combustible energy of that part of the carbohydrates which becomes converted to micro-organisms is apparently well utilized by the animal, for there is ample evidence that micro-organisms which proliferate in the paunch are well digested and are absorbed when they pass to lower levels of the tract. A considerable proportion of this fraction, however, would be represented as protein, and so would be subject to the usual losses associated with protein metabolism.

The products of the rumen fermentation of carbohydrates differ widely in their usefulness to the animal. Methane is expelled and so its combustible energy will be a total loss. Butyric acid might be expected to be utilized efficiently in the fat cycle. The whole of the combustible energy of the propionic acid is probably capable of supporting the processes involved in the maintenance, growth and production of the animal; propionic acid is readily convertible to glucose (Ringer, 1912; Deuel, Butts, Hallman & Cutler, 1935–6) and so would enter the carbohydrate cycle. Acetic acid, however, is by no means so adaptable in metabolism. While there is a great body of evidence which indicates that in the animal it is not convertible to carbohydrate precursors (Ringer & Lusk, 1910; Ringer, 1913; Deuel & Milhorat, 1928; Ponsford & Smedley-MacLean, 1932; Stöhr, 1933; Deuel *et al.* 1935–6; Cross & Holmes, 1937), it is not improbable from more recent evidence that the fat and carbohydrate cycles may be linked through 2-C compounds. The ease and versatility of carbon dioxide fixation, however, renders difficult the assessment of energy costs incurred during the transfer of acetic acid into productive channels. As carbon dioxide labelled with isotopic carbon finds its way into glycogen (Solomon, Vennesland, Klemperer, Buchanan & Hastings, 1941; Buchanan, Hastings & Nesbett, 1942; Vennesland, Solomon, Buchanan & Hastings, 1942) the interpretation of the mechanism, through which the carbon isotope from C^{13}-carboxyl-tagged acetate is transferred to glycogen, is complicated, and as yet there is no general agreement as to the course it takes (Buchanan *et al.* 1943; Lorber, Lifson & Wood, 1945).

If acetic acid is rapidly oxidized and its combustible energy dissipated as heat without entering into the productive channels of metabolism, its combustible energy must be classed with the heat evolved in the exothermic metabolic processes of the icro-organisms responsible for the dissimilation of arbohydrate in the rumen, for it would serve only spare other foodstuffs when the animal is in a ld environment. If acetic acid enters the carbo-ydrate cycle without appreciable loss, its com-ustible energy should be classed with that of pro-ionic and butyric acids.

In the former case the products of the *in vitro* ermentations of cellulose would have for the animal productive value between 67 and 72 % of the com-ustible energy of the cellulose fermented (i.e. ·8–3·0 kg.cal./g. cellulose); in the latter, if acetic cid follows a course similar to that of propionic cid or butyric acid and enters without loss into ither the carbohydrate or fat cycle, the productive alue of the fermentation products would be be-ween 85 and 88 % of the combustible energy of the ellulose (i.e. 3·6–3·7 kg.cal./g. cellulose).

The problem of the comparative usefulness of cetic and propionic acids in ruminant nutrition has een investigated through the direct approach of nimal calorimetry. Experiments to determine the apacity of acetate and propionate to provide nergy, to spare tissue destruction in the fasting heep and to promote a positive energy balance when added to maintenance rations will be discussed lsewhere.

SUMMARY

1. Under environmental conditions similar to hose in the normal rumen, cellulose, when fermented *in vitro* by associative growths of micro-organisms eparated from actively fermenting rumen contents, yielded acetic acid, propionic acid, carbon dioxide and methane as main products, together with smaller quantities of formic acid and butyric acid, and traces of acetaldehyde, pyruvic acid and lactic acid.

Propionic acid predominated among the dissimi-lation products; the molecular ratio of propionic to acetic in four digests ranged from 1·5 to 1·8. The molecular ratios of carbon dioxide to methane ranged from 3·0 to 3·8.

2. Depending on the duration of the fermentation the amount of combustible energy in the micro-organisms which proliferated ranged between 7 and 16 % of that of the cellulose fermented. The cost of production and maintenance of these organisms, estimated from the carbon and energy balance sheets, was approximately 6 % of the combustible energy of the cellulose fermented. As the relationship between the carbon and combustible-energy contents of the micro-organisms and that of the cellulose is approximately the same (9·4 kg.cal./g. C), the energy dissipated as heat during fermentation was close to the energy deficit of the products of fermentation, viz. 6 % of the combustible energy of the cellulose fermented.

3. The energy metabolism of the ruminant is discussed in light of these findings, and it is concluded that the useful energy of the dissimilation products would be between 85 and 88 % of the combustible energy of the cellulose (i.e. between 3·6 and 3·7 kg.cal./g. cellulose) if the acetic acid enters without loss into productive channels of metabolism, and between 67 and 72 % of the combustible energy of the cellulose (i.e. between 2·8 and 3·0 kg.cal./g. cellulose) if the acetic acid is oxidized and wastefully dissipated. These figures would apply to the ruminant in a warm environment; under cold conditions both heat evolved during the dissimilation and the combustible energy of the acetic acid, irrespective of whether it is otherwise useful to the animal, would be available to spare other nutrients which would be oxidized to maintain body temperature.

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The Estimation of Suramin in Plasma

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The colorimetric estimation of suramin (Antrypol, Bayer 205) by conversion of the hydrolytic fission products to an azo dye has been described by several investigators. Dangerfield, Gaunt & Wormall (1938) have summarized the earlier work, and have described in detail a method in which the drug is subjected to acid hydrolysis; the fragments are then diazotized and coupled with methyl-α-naphthylamine. Spinks (1948) has used alkaline hydrolysis and *N*-*β*-sulphatoethyl-*m*-toluidine as a dyestuffs end component. Balaban & King (1927), in their investigation of the affinity of suramin and other complex ureides for cellulose, devised a roughly quantitative method in which the concentration was determined from the turbidity produced by the addition of a basic protein such as clupeine sulphate; for this method the authors claimed a sensitivity of 1 in 100,000.

In this investigation the possibility of a direct, colorimetric estimation by a dye-laking procedure has been explored by examining the behaviour of a range of basic dyes in the presence of the drug. Certain styrylquinoline dyes have been found to exhibit a marked colour change under these conditions, and 2-*p*-dimethylaminostyryl-6-acetamidoquinoline methochloride has been selected as the most suitable for the estimation. This compound was described by Browning, Cohen, Ellingworth & Gulbranson (1926); it is 'styrylquinoline 24' in their series and will be referred to here as *SQ* 24.

In the presence of excess suramin the deep-red colour of *SQ* 24 is converted to a pale yellow; the change in absorption spectrum is shown in Fig. 1.

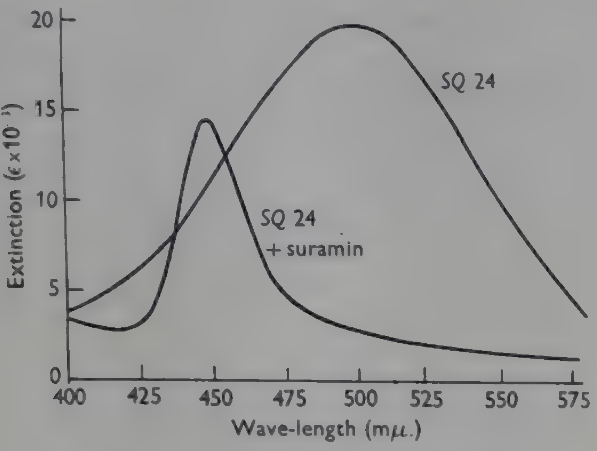


Fig. 1. Absorption spectra of *SQ* 24, with and without added suramin.

SQ 24 has a broad maximum at 495 mμ., and the complex with suramin has a sharp maximum at 450 mμ. The greatest difference between the two curves is to be found at 505 mμ. The complex cannot be extracted by organic solvents, and on standing overnight the yellow aqueous solution deposits a flocculent brown precipitate. If a sufficient concentration of water-miscible organic solvent, such as ethanol or cellosolve, is added to the yellow solution the original red colour of the reagent is regenerated. Plasma proteins do not interfere with the suramin reaction, and the concentration of the drug in plasma may be determined by comparing the optical density of the dye solution, to which has been added diluted plasma (in sufficient 0·9 % NaCl to prevent globulin precipitation), with that of the dye solution without suramin.

METHOD

The analytical procedure described below for plasma is suitable for serum or for aqueous solutions of suramin. The quantities given are for plasma samples containing 0·5–15 mg./100 ml.; for greater or lesser amounts of suramin the ratio of sample to dye solution should be suitably modified.

Reagents required. (*a*) 2-*p*-dimethylaminostyryl-6-acetamidoquinoline methochloride (*SQ* 24) solution; 1 mg./100 ml. distilled water. (*b*) Isotonic sodium chloride (0·9 % NaCl in water).

Apparatus required. (*a*) 6 × ¾ in. test tubes with graduation mark at 10 ml. (*b*) Suitable colorimeter. If none is available, an approximate estimate of the suramin concentration may be made by comparison with a set of freshly prepared standards. In this investigation the Coleman spectrophotometer at a wave length 505 mμ. has been used employing cylindrical cells. Satisfactory results have also been obtained with the Spekker absorptiometer using an Ilford 603 blue-green filter and 1 cm. cells. To avoid adsorption of the dye on the surface of glassware it is important that the tubes and colorimeter cells should be free from surface etching; they should be well cleaned in chromic-sulphuric acid mixture before use.

Procedure

(*a*) Pipette 0·2 ml. plasma into 2 ml. isotonic NaCl solution in a tube graduated at 10 ml. Add 5 ml. *SQ* 24 solution and make up to 10 ml. with distilled water.

(*b*) Pipette 5 ml. *SQ* 24 solution into 2 ml. isotonic NaCl and make up to 10 ml. with distilled water.

(*c*) Pipette 0·2 ml. plasma into 2 ml. isotonic NaCl and make up to 10 ml. with distilled water. The solutions are allowed to stand for a few minutes and the optical densities measured in a suitable colorimeter. The density of the plasma blank (*c*) is subtracted from that of (*a*), and the difference subtracted from the dye blank (*b*). The amount of suramin present in (*a*) may then be determined by applying this difference to a standard curve, constructed from solutions containing known amounts of suramin. Fig. 2 shows standard curves constructed by the above method; curve *A* is for dilutions of suramin in water, while to obtain curve *B* the solutions contained a total concentration of 20 % (v/v) serum. With the amounts of plasma or serum used in the method as described above, the standard curve coincides with the water curve *A*.

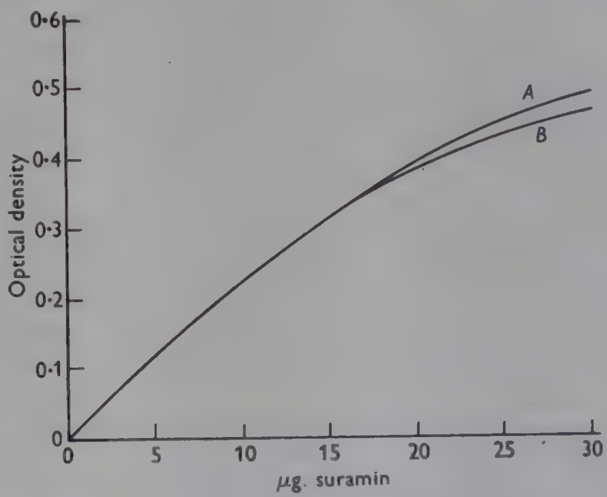


Fig. 2. Standard curve for suramin estimation, *A* in water, *B* in 20 % serum.

DISCUSSION

Comparison with the hydrolysis method

Samples of oxalated plasma, taken at intervals from a rabbit dosed intravenously with 100 mg./kg. of suramin, were subjected to the above analytical procedure and also to the hydrolysis method of Dangerfield *et al.* (1938), modified by the use of the Rose & Bevan (1944) end component *N*-β-sulphatoethyl-*m*-toluidine. The results by the two methods are compared in Table 1.

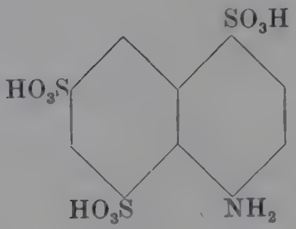
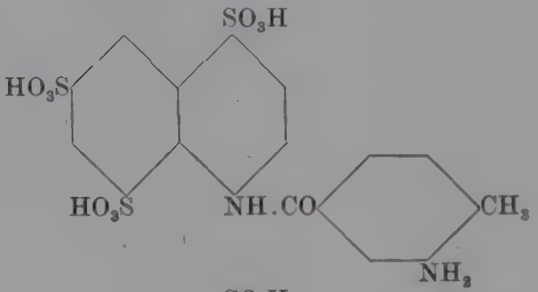
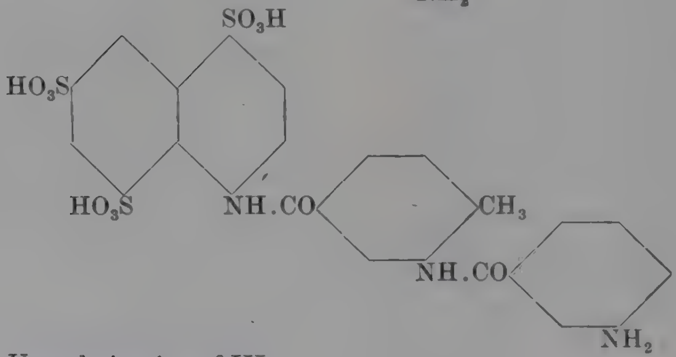
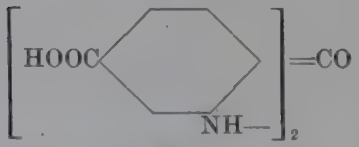
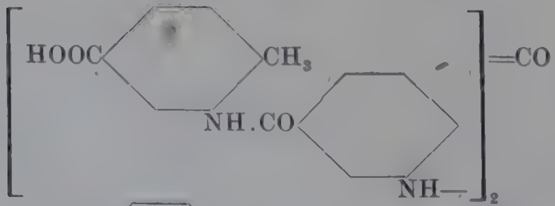
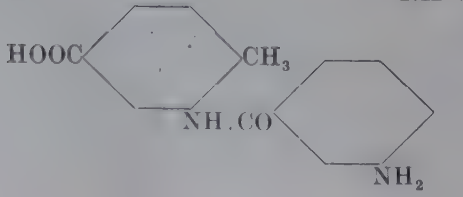
Table 1. *Concentration of suramin in the plasma of an injected rabbit, by dye-laking and hydrolytic methods*

	Plasma suramin (mg./100 ml.)	
Time after dosing	Dye-laking	Hydrolysis
15 min.	71·2	61·6
5 hr.	25·2	21·6
1 day	16·2	15·2
4 days	6·1	5·7
10 days	2·4	2·5
20 days	2·3	3·2

The blank value of the rabbit plasma prior to dosing was 1·3 mg. by the dye-laking method, and 0·96/100 ml. by the hydrolysis method.

Since the dye-laking method necessitates the measurement of a colour difference it might be expected to be rather less precise that the hydrolysis method, particularly with the lower suramin concentrations. The magnitude of this colour difference, however, makes the dye-laking method rather more sensitive; together with the far greater manipulation necessary for the hydrolysis method, this

Table 2. *Suramin 'equivalents' of possible metabolic fission products*

	Formula	Mol. compound equivalent to 1 mol. suramin
I		0·20
II		0·41
III		0·31
IV	Urea derivative of III	0·84
V		0·20
VI		0·24
VII		0

probably makes the precision of the two methods of about the same order. The very much simpler technique of the dye-laking procedure, and the shorter time required, should make the method of particular value if plasma concentrations are required to be estimated in the field with limited laboratory facilities.

Specificity of the dye-laking method

A number of the possible metabolic fission products of suramin have been subjected to the analytical procedure described above and the results are listed in Table 2.

These results indicate that the dye-laking reaction occurs with those fragments which contain the naphthylaminetrisulphonic acid group and also the bis-urea derivatives. It may be assumed from the results with (VII) that amides and the simpler amino-acids do not give the reaction. It is interesting to note that the order of suramin equivalents of this series of compounds approaches very closely to their order of persistence in the blood stream as found by Spinks (1948).

Table 1 indicates that the results obtained by the two methods are of the same order, the dye-laking method giving slightly the higher figures. This suggests the absence from the plasma of appreciable amounts of simple amino-acids and amides without the naphthylaminetrisulphonic acid group, since these would cause the dye-laking method to give lower apparent plasma concentrations. On the other hand appreciable amounts of naphthylaminetrisulphonic acid would cause the dye-laking method to give a higher apparent concentration, since according to Spinks (1948) the azo dye developed from this compound is weakly coloured. It is possible that the observed higher results with the dye-laking method are due to this cause. Spinks has also shown that persistence for a prolonged period in the blood after intravenous injection is only exhibited by symmetrical polyamides of high molecular weight, which contain naphthylaminepolysulphonic acids as end groups, and that after 10 hr. the amounts of the other products of hydrolysis of suramin in the blood are negligible. Moreover, since all the degradation products examined have no trypanocidal activity, it is very likely that after this period both methods are measuring true suramin concentration and any divergence between them is due to experimental error.

SUMMARY

1. A method for estimating suramin in aqueous solution or in serum and plasma is described. It utilizes the colour change which is observed when suramin is added to a styrylquinoline dye, 2-*p*-dimethylaminostyryl-6-acetamidoquinoline methochloride.

2. The method is of about the same precision as the hydrolysis method of Dangerfield *et al.* (1938) and rather more sensitive; it involves a much simpler and more rapid technique. The two methods yield similar values for the suramin content of the blood of a rabbit injected with suramin.

3. The behaviour of hydrolysis products of suramin has been investigated and the specificity of the method is discussed.

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Sexual Differences in the Storage and Metabolism of Iron

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It is now an accepted fact that hormones and/or vitamins have important parts to play in the exchanges and functions of sodium, chlorine, potassium, calcium and iodine, but their role in the metabolism of iron has not been so conclusively demonstrated or so generally recognized. Bunge (1889) suggested that young female animals put aside reserves of iron for the future needs of reproduction, and that the deviation of iron from the circulation to the storage organs at puberty might explain the high incidence of chlorosis in adolescent girls. In more recent years further evidence has been produced that the metabolism of iron is controlled, to some extent at any rate, by the sex hormones. Women, for instance, tend to have lower concentrations of circulating haemoglobin than do men, apart altogether from the obvious effects which menstruation must have upon their bodily requirements (Widdowson & McCance, 1936, 1942; Barer & Fowler, 1936). There is also good evidence that they have less inorganic iron in the serum (Vahlquist, 1941). Pregnancy depletes the iron stores of female rats (Smythe & Miller, 1929; Steenbock, Semb & Van Donk, 1936), and Mitchell (1932) showed that if males and females were given a diet which was deficient in iron the males became anaemic more quickly than the females. Smith & Otis (1937) found that, given the same diet, female rats produced more haemoglobin/g. of available iron than did males, and Steenbock *et al.* (1936) that they accumulated more iron in their livers. Hubbell & Rose (1937) and Smith & Otis (1940) showed that the whole bodies of female rats contained more iron/g. than those of males given similar amounts of iron in their diets; Smith & Otis (1940) further demonstrated that, at a low level of intake, the total amount of iron in the bodies of the females was greater than that in the bodies of the males, despite the fact that the females were smaller animals. Steenbock *et al.* (1936), moreover, showed that castration decreased a female's capacity to store iron, and that injections of follicular hormone into immature females stimulated them to store more iron than their litter mates. This work was reported very briefly and no confirmation or extension of it has been found. It is evident, however, that both in the rat and in man there are quantitative differences in the way in which the two sexes use the iron available to them.

The experiments now to be described were undertaken to confirm some of these findings and to discover to what extent they were true of other species. The work formed part of a more general study of the chemistry of growth and development. A short communication has already been made to the Journées thérapeutiques de Paris (McCance & Widdowson, 1946).

METHODS

Rats, mice, rabbits, guinea pigs, poultry, perch and eels have been used for these experiments. The rats were an inbred black and white strain, and were reared on a stock diet consisting of bread, oats, bran, fish meal, milk and greenstuff. The mice, which were highly inbred, were given similar food, and the rabbits and guinea pigs their usual diets. The mice, rabbits and guinea pigs were obtained from the Pathological Laboratory, Cambridge. The cockerels and pullets were specially reared for the experiment by Chivers and Sons Ltd. They were Light Sussex, and the numbers of females and males were the same. The two sexes received exactly the same diet. The perch and eels were obtained from the Freshwater Biological Station, Wray Castle, Windermere. They had not been reared in captivity.

Total iron was determined after dry ashing by the thioglycollic acid method (McCance, Widdowson & Shackleton, 1936). Inorganic iron in the liver was estimated by the modification of Hill's (1930) method described by Shackleton & McCance (1936).

RESULTS

The effects of age, sex, and reproduction on the concentration of inorganic iron in the livers of rats

Seventy-eight male and 99 female animals were used for these experiments, and the results are shown graphically in Fig. 1. It will be seen that the livers of newborn animals contained between 14 and 15 mg. of inorganic iron/100 g. of wet tissue. At the end of a fortnight the inorganic iron had fallen to less than 1 mg./100 g. of liver. This was an expression of the deficiency of iron to which all mammals are liable towards the close of the suckling period. In rats this is not usually accompanied by anaemia, nor does it affect one sex more than another. By the time the young rats were 50 days old the concentration of inorganic iron in the livers of both sexes had risen to about 4 mg./100 g., but with the onset of sexual development the females began to lay down iron in their livers much faster than did the males, and by the time the animals were 90 days old and sexually mature, the female livers contained 17 mg. whereas the male livers contained but 5 mg./100 g. At 200 days the iron in the female livers had reached a level of 34 mg. and in the male livers 11 mg./100 g., so the difference had continued to increase. At this age some of the female animals were mated and at the end of pregnancy the liver iron was found to have fallen to little more than 8 mg./100 g. No increase took place during lactation, but by the time the surviving mothers had reached the age of 320 days, their livers contained 18 mg./100 g. This was a greater concentration of iron than was found in male livers at 320 days, but a lower concentration than was found in the livers of the unmated females.

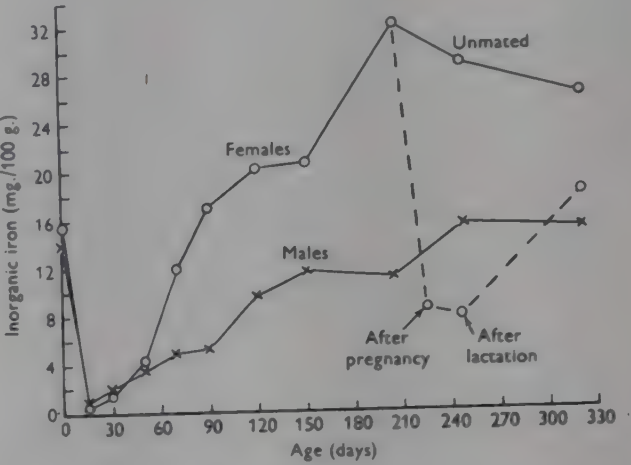

Fig. 1. Concentration of inorganic iron in the livers of male and female rats.

It is evident that the stores of iron in the livers were drawn upon freely to supply the needs of the foetuses, and the present results can be used to show the extent to which they did so. The liver of an unmated female rat 200 days old contained about 3·8 mg. of inorganic iron. By the end of pregnancy this may be considered to have fallen to 1·1 mg., a difference of 2·7 mg. The bodies of the newborn rats each contained about 0·36 mg. of iron, and, assuming a litter to consist of 8 young animals, this suggests that during pregnancy each mother had to provide 2·8 mg. of iron for her progeny. This is very close to the amount which disappeared from the liver, and suggests that most of the iron in the newborn animals may have come from that source. It would have been interesting to have studied the effects of a second pregnancy, but there were not sufficient animals to enable this to be done.

The effects of castration and sex hormones upon the concentration of inorganic iron in the livers of rats

Eighteen male and 24 female rats were used for these experiments, and they were all killed at the age of 90 days, which is about the time at which rats become sexually mature. The males were divided into three groups. The animals in group 1 were allowed to grow normally, those in group 2 were castrated at the age of 46 days; those in group 3 were also castrated at the same age, but were subsequently treated with subcutaneous injections of stilboestrol; 0·3 mg. of stilboestrol dissolved in arachis oil was injected each day for 3 days, the injection was stopped for 2 days, given for the next 3 days and so on. The females were divided into four groups. The animals in group 1 were allowed to grow normally, and those in group 2 were spayed at the age of 46 days. Those in groups 3 and 4 were also spayed at the same time, but the animals in group 3 were subsequently given stilboestrol by injection, 0·3 mg. a day for 3 days, none for 2 days, the injection continued for 3 days and so on. In this way it was possible to establish a regular oestrous cycle, which was followed by means of vaginal smears. The animals in group 4 were given 0·25 mg. of testosterone each day, also by subcutaneous injection.

The livers of each group were pooled for analysis, and the results are given in Table 1. It will be seen that removing the gonads made the two sexes grow at the same rate, equalized the weights of their livers, and the concentration of inorganic iron in them. The administration of stilboestrol to the castrated males did not increase the percentage of iron in their livers, but it did increase slightly the percentage of iron in the livers of the spayed females. Testosterone did not appear to decrease the percentage of iron in the livers of such animals, or if it did the effect was small. Changes might have been produced by administering larger doses of stilboestrol and testosterone to the male and female castrates respectively, but the present results, taken at their face value, suggest that the male and female hormones may normally act in conjunction with factors present only in bodies of the same sex, for there can be no doubt about the effects of the natural hormones; there are, moreover, the findings of Steenbock *et al.* (1936), to which reference has already been made, that the injection of follicular hormones into immature females increased their capacity to store iron in their livers.

The effects of sexual development on the concentration of inorganic iron in the livers of other animals

Mice. Twelve adult male and 18 unmated female animals were killed. Their livers were removed and those of each sex were pooled for analysis. 1·08 mg. of inorganic iron was found in each 100 g. of male liver and nearly three times as much (3·08 mg./100 g.) in the female organ.

Guinea pigs. Six adult male and 6 adult unmated female animals were killed and the livers were worked up for analysis as already described. The male livers contained 4·9 mg. and the female 4·1 mg. of inorganic iron/100 g.

Table 1. *Effect of castration and of sex hormones on the concentration of iron in the livers of sexually mature rats* (90 *days*)

	Body wt. (g.)	Liver wt. (g.)	Liver as % body wt.	Inorganic Fe in liver Total/rat (mg.)	Inorganic Fe in liver mg./100 g.
Males					
No treatment	296	12·7	4·3	0·88	6·9
Castrated	258	9·5	3·7	1·05	11·1
Castrated, and stilboestrol	241	9·4	3·9	0·96	10·2
Females					
No treatment	199	7·3	3·7	1·32	18·2
Spayed	258	10·3	4·0	1·29	12·5
Spayed, and stilboestrol	222	8·8	4·0	1·36	15·5
Spayed, and testosterone	242	9·8	4·0	1·06	10·7

Rabbits. Five male and 6 female animals were killed at the age of 17–25 weeks. The liver of each animal was analyzed separately. Those of the males were found to contain an average of 10·3 mg. and the female livers an average of 8·2 mg. of inorganic iron/100 g.

Eels. Twelve silver males and 11 silver females were caught on their way to the sea; their average weights were 75 and 347 g. respectively. The livers of each sex were pooled for analysis. Those of the males held 28·2 mg. and those of the females 83·5 mg. of inorganic iron/100 g. This figure for inorganic iron in the livers of silver females, although extremely high, is about the value which might have been anticipated from the findings of McCance (1944) for the total iron in the livers of sexually mature females.

Perch. The livers of 11 immature males and of 11 immature females were pooled for analysis. The male fish averaged 24 g. and the females 19 g. in weight. The livers of the former contained 7·5 and of the latter 7·6 mg. of inorganic iron/100 g. Twenty-four male fish and 14 females were caught in May when they were ripe. The male and female fish then weighed 23 and 41 g. respectively. Analysis revealed 9·6 mg. of inorganic iron/100 g. in the male livers and 9·5 mg./100 g. in the female livers. These figures show that the development of the sperm and ova did not take place at the expense of iron stored in the liver.

Poultry. Twelve cockerels and 12 pullets were killed when they were 20 weeks old. Their livers were analyzed separately, and the results are given in Table 2. The average for the cockerels was 8·7 mg. and for the pullets 14·7 mg./100 g. The difference was statistically significant ($t=2{\cdot}49$, $P=0{\cdot}05$–$0{\cdot}02$). The male spleens contained 4·7 mg. and the female spleens 8·5 mg. of inorganic iron/100 g., so sex appeared to affect the storage of iron in this organ also.

Table 2. *Concentration of inorganic iron in the livers of cockerels and pullets*

	Liver inorganic Fe (mg./100 g.)	
Bird no.	Cockerels	Pullets
1	8·7	10·5
2	7·0	37·3
3	6·1	20·3
4	11·1	12·8
5	7·0	10·7
6	8·4	20·2
7	9·6	11·5
8	10·3	12·2
9	9·9	10·3
10	13·4	7·0
11	6·0	10·1
12	6·4	13·4
Average	8·7	14·7

DISCUSSION

The results obtained with rats have confirmed those of previous workers, and there can be no doubt at all that sex has an important influence on the storage of iron in this animal. The same is probably true of mice, but the concentrations of iron are very much lower in this species, so much so that amounts stored can scarcely be of any practical value to either sex. Since, however, sex does not seem to influence the storage of iron in either guinea pigs or rabbits, it is clear that even within the mammalian order these sex differences do not always appear, and they can certainly not be taken to hold for any given species until their presence has been experimentally verified. It will be exceedingly difficult to do this for the human species. While, therefore, these differences can never be ranked among the great biological generalizations, it is stimulating to have found them so easily in each of the three vertebrate orders which have been investigated. It is clear that a great deal more work will have to be done to find out how the sex hormones bring about these effects, and why they do not always do so, for the sex hormones of the rat must be very similar to those of the rabbit or indeed of any other vertebrate. What can the second factor be which enables the sex hormones to exert their effects, or which prevents them doing so? It may be unwise to formulate theories at this stage, but it can do no harm to point out that the female rat has been shown to store vitamin A more readily than the male under identical conditions of feeding and whether carotene or vitamin A is acting as the source of supply. Yet males have higher blood levels (Brenner, Brookes & Roberts, 1942). During periods of depletion, moreover, male rats were found to lose their stores of vitamin A more readily than female rats (Popper & Brenner, 1942). Is this association of iron and vitamin A wholly fortuitous? Few biological discoveries can be fitted into their proper place in the framework of knowledge until some time after they have been made, and they often turn out to have a much more general application than was at first supposed.

SUMMARY

1. With the onset of sexual development, female rats have been found to accumulate iron in their livers at a much greater rate than males.

2. Removal of the gonads from male and female rats made the males store more and the females less iron in their livers, so that the concentrations were equal for the two sexes. The administration of stilboestrol had no effect on the castrated males, but it caused a slight increase in the iron storage of the spayed females.

3. The livers of sexually mature female rats, mice, hens and eels have been shown to have a greater concentration of iron in them than the livers of male animals at a corresponding stage of development.

4. Guinea pigs, rabbits and perch were not found to exhibit these sex differences.

5. Pregnancy rapidly reduced the percentage of iron in the livers of female rats and the reaccumulation was comparatively slow.

The authors wish to thank those who have assisted them to obtain the animals used in this investigation, and Miss E. Wilkinson for operating on the rats. The work was made possible by the help given to the Department by the Medical Research Council.

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A Synthesis of *dl*-Hydnocarpic Acid

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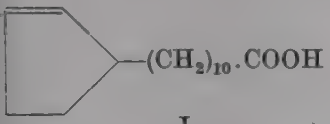
(*Received* 13 *October* 1947)

Hydnocarpic acid in its dextro-form is isolated from several oils (e.g. oil of *Hydnocarpus wightiana*) used in the treatment of leprosy. The structure (I) assigned to hydnocarpic acid rests on the results of permanganate oxidation (Barrowcliff & Power, 1907), the synthesis of dihydrohydnocarpic acid (Nöller & Adams, 1926*a*; Bokil & Nargund, 1937), the homologation to and from chaulmoogric acid (II) (Stanley & Adams, 1929; Buu-Hoï, 1944) and a synthesis of *dl*-hydnocarpic acid by a route involving several changes in a *cyclo*pentane ring (Bokil & Nargund, 1941).



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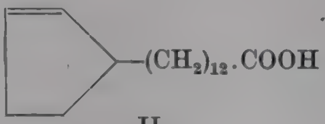
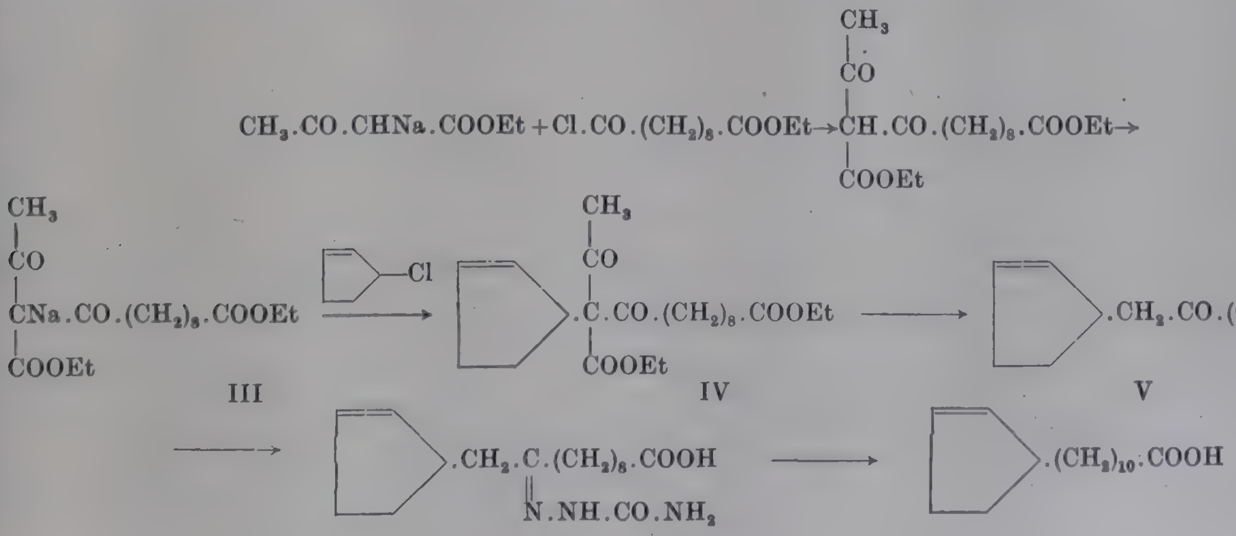


II

The synthesis of *dl*-chaulmoogric acid (II), at the Culion Leper Colony (Perkins & Cruz, 1927*b*) is now a classic, and it was thought worth while to apply the same general method (the Robinson long-chain synthesis, Robinson & Robinson, 1925) to the synthesis of hydnocarpic acid.


CH₃.CO.CHNa.COOEt + Cl.CO.(CH₂)₈.COOEt → CH₃.CO.CH(COOEt).CO.(CH₂)₈.COOEt →
CH₃.CO.CNa(COOEt).CO.(CH₂)₈.COOEt
III
Cl
.C(CO.CH₃)(COOEt).CO.(CH₂)₈.COOEt
IV
.CH₂.CO.(CH₂)₈.COOH
V
.CH₂.C(=N.NH.CO.NH₂).(CH₂)₈.COOH
.(CH₂)₁₀.COOH

The ester-acid chloride of sebacic acid reacts smoothly with ethyl sodioacetoacetate and the product yields a sodio derivative (presumably III). *cyclo*Pent-2-enyl chloride, as a substituted allyl chloride (Noller & Adams, 1926*b*), reacts in the cold with (III) to give the complex (IV); this complex is hydrolyzed, mainly with the loss of the acetyl and the carbethoxy groups, to 10-ketohydnocarpic acid (V). This keto acid is isolated as its semicarbazone which, on heating with sodium ethoxide, yields *dl*-hydnocarpic acid. This specimen of synthetic *dl*-hydnocarpic acid did not depress the melting point of the natural *d*-acid; Hinegardner (1933) found that *dl*-hydnocarpic acid, obtained by racemization of hydnocarpamide, did not depress the melting point of the *d*-acid. Confirmation follows from the preparation of the dihydro derivatives in which there is no asymmetry. The results are summarized in Table 1.

Table 1. *Melting points of natural and synthetic acids and derivatives*

	Found		
	Synthetic *dl*	Natural *d*	References
Hydnocarpic acid	59– 59·5°	59·5– 60°	*dl* 58·5–59° (Hinegardner, 1933) *dl* 58–59° (Synthetic; Bokil & Nargund, 1941) *d* 59–60° (Power & Barrowcliff, 1905) *d* 60·5° (Cole & Cardoso, 1937)
Hydnocarpamide	108·5–109°	109–109·5°	*dl* 109–110° (Synthetic; Bokil & Nargund, 1941) *d* 111–112° (Hinegardner, 1933)
Dihydrohydnocarpic acid	62·5– 63°	63°	63–63·5° (Noller & Adams, 1926*a*) 63° (Wrenshall & Dean, 1924)
Dihydrohydnocarpamide	114·5–115·5°	114·5–115·5°	114–115° (Noller & Adams, 1926*a*)

EXPERIMENTAL

9-Carbethoxynonoyl chloride. Ethyl hydrogen sebacate, prepared from diethyl sebacate by the procedure of Grün & Wirth (1922) and crystallized from light petroleum (b.p. 60–80°) melted at 36·5°. (Found: C, 62·3; H, 9·6%. Calc. for $C_{12}H_{22}O_4$: C, 62·6; H, 9·6%.) Refluxing for 4 hr. with excess of thionyl chloride gave carbethoxynonoyl chloride as a viscous oil, b.p. 141–144°/0·14 mm., or 168–170°/14 mm. (yield 80%). By the action of ice-cold aqueous ammonia *9-carbethoxynonamide*, m.p. 68·5–69°, was prepared. (Found: C, 63·2; H, 9·9; N, 6·7%. $C_{12}H_{23}O_3N$ requires C, 63·0; H, 10·0; N, 6·2%.)

Ethyl 10:12-diketo-11-carbethoxytridecoate. Sodium (2·3 g., 1 equiv.) was powdered under xylene and the powder washed with dry toluene. Dry toluene (70 ml.) was added and then, with cooling, 13 g. (1 mol.) of redistilled ethyl acetoacetate; the mixture was allowed to stand with occasional shaking until all the sodium had reacted (1 hr.).

Freshly prepared 9-carbethoxynonoyl chloride (24·8 g., 1 mol.) in toluene (20 ml.) was then added slowly with shaking and after half an hour the mixture was boiled under reflux until it became neutral to litmus (30 min.). The resulting ethyl 10:12-diketo-11-carbethoxytridecoate was not isolated: its sodio derivative reacted smoothly with *cyclo*pent-2-enyl chloride.

Ethyl 11-(cyclopent-2-enyl)-11-carbethoxy-10:12-diketotridecoate. The cold, toluene solution of the ester obtained above was added slowly to powdered sodium (2·3 g., 1 equiv.) covered with toluene, and the mixture was boiled under reflux for 2 hr., when almost all the metal had reacted. A freshly prepared solution of *cyclo*pent-2-enyl chloride (Perkins & Cruz, 1927*a*; 12·8 g., 1·25 mol.) in toluene (11 ml.) was added: NaCl separated and the mixture became pasty. After refluxing for 1 hr. it had an acid reaction to phenolphthalein. With the aid of water (50 ml.) containing sodium acetate crystals (0·5 g.) the gelatinous mixture was transferred to a 500 ml. flask and steam was passed to destroy or remove *cyclo*pentenyl chloride, *cyclo*pentadiene and toluene. The residual ester was collected with the aid of ether; a sample of it boiled at 180–190°/0·04 mm.; it had $n_D^{20^\circ}$ 1·4511 and gave a red-brown colour with ethanolic $FeCl_3$ solution.

Semicarbazone of 10-ketohydnocarpic acid. Hydrolysis of the crude ethyl 11-(*cyclo*pent-2-enyl)-11-carbethoxy-10:12-diketotridecoate (31 g., 1 mol.) in ethanol (50 ml.) was carried out by slowly adding aqueous KOH (24 g. in 30 ml. of water) with vigorous shaking; potassium salts were precipitated and much heat evolved. Water (50 ml.) was added to give a clear brown solution which was boiled for 1 hr. under reflux: a test portion was then miscible with water. Steam was passed through the solution to remove ethanol and *cyclo*pentenylacetone.

Acidification of the alkaline residue in the distilling flask with conc. HCl (50 ml.) precipitated a brown mixture of solid acids (18 g., dry) from which, by rapid distillation at 0·5 mm. pressure, 15 g. of colourless waxy solid, m.p. 85–102°, were obtained. This solid was a mixture mainly of sebacic and 12-ketohydnocarpic acids which could not easily be separated by crystallization. The mixture of acids (15·3 g.) in ethanol (50 ml.) was added to a solution of semicarbazide hydrochloride (5 g.) and hydrated sodium acetate (6·5 g.) in water (50 ml.). After the resulting mixture had been warmed for 15 min. on the water bath it was allowed to stand overnight: 11·0 g. of colourless prisms, m.p. 125–127°, were deposited. (Yield 28%, calculated on the ethyl 11-(*cyclo*pent-2-enyl)-11-carbethoxy-10:12-diketotridecoate.) Recrystallized from ethanol and then from *n*-propyl alcohol-benzene the substance melted at 135·5–136·5°. (Found: C, 63·0; H, 9·1; N, 12·8%; equivalent, by titration, 318. $C_{17}H_{29}O_3N_3$ requires C, 63·1; H, 9·0; N, 13·0%; equivalent, 323.)

dl-Hydnocarpic acid. The purified semicarbazone (3·7 g., 1 mol.) was heated with sodium ethoxide (from 0·77 g. Na, 3 equiv.) in ethanol (9 ml.) at 200° for 21 hr. After acidification the product yielded to ether 2·2 g. (76% yield) of yellow solid. Rapid distillation at 0·1 mm. followed by crystallization from light petroleum-ethyl acetate gave pearly plates of m.p. 59–59·5°. Hinegardner (1933) gave m.p. 58·5–59° for the racemic acid. (Found: C, 76·4; H, 11·1%; equivalent by titration, 248. Calc. for $C_{16}H_{28}O_2$: C, 76·2; H, 11·1; equivalent, 252.) The amide prepared by the usual method melted at 108·5–109°.

Dihydrohydnocarpic acid (*synthetic*). The *dl*-hydnocarpic acid (0·3 g.) in ethanol (10 ml.) was reduced with hydrogen at 1 atmos. pressure in presence of Raney nickel. In 50 min. 28 ml. of hydrogen (at 756 mm. and 20°) were absorbed (theory, 27·5 ml.) and the product (0·25 g.) melted at 59–60°. Crystallization from light petroleum-ethyl acetate gave pearly plates of m.p. 62·5–63°. (Found: C, 75·5; H, 11·9%. Calc. for $C_{16}H_{30}O_2$: C, 75·6; H, 11·8%.) The amide, obtained in the usual way and crystallized from ethanol, melted at 114·5–115·5°. (Found: C, 75·9; H, 12·2%. Calc. for $C_{16}H_{31}ON$: C, 75·9; H, 12·3%.)

d-Hydnocarpic acid (*from Hydnocarpus oil*). Oil of *Hydnocarpus wightiana* (40 g.) was boiled for 5 hr. under reflux with ethanolic KOH solution (128 ml. of 2·5 N). Water (50 ml.) was added and, after heating had been continued for a further 10 hr., acidification gave a yellowish, waxy mixture of acids (36 g.). Esterification of these acids by boiling for 21 hr. with ethanol (56 ml.) and H_2SO_4 (3 ml.) gave on cooling a dark layer of ethyl hydnocarpate and ethyl chaulmoograte, sparingly soluble in ethanol (Buu-Hoï, Cagniant & Janicaud, 1941). The ester layer was diluted with ether, washed with water, Na_2CO_3 solution, with water and then dried: it yielded 31 g. of mixed ethyl esters. Repeated fractional distillation of these esters with the aid of a Vigreux column gave 6·1 g. of ethyl hydnocarpate (b.p. 164–165°/0·6 mm., $n_D^{20°}$ 1·4583). (Found: C, 77·0; H, 11·5%. Calc. for $C_{18}H_{32}O_2$: C, 77·1; H, 11·4%.) The ethyl chaulmoograte fraction boiled at 172–174°/0·6 mm., $n_D^{20°}$ 1·4600. Hydrolysis of the ethyl hydnocarpate by refluxing with ethanolic KOH (10% (w/v) KOH, 10% (v/v) water) for 4·5 hr. gave 5·5 g. of crude acid, m.p. 51–54°. Recrystallization from light petroleum-ethyl acetate gave colourless leaflets, m.p. 59·5–60° alone or when mixed with the specimen of synthetic *dl*-acid. The amide, crystallized from ethanol, melted at 109–109·5° and did not depress the m.p. of a specimen of the synthetic *dl*-amide (m.p. 108·5–109°).

Dihydrohydnocarpic acid (*from the d-acid*). Pure *d*-hydnocarpic acid (0·45 g.) dissolved in ethanol absorbed 43 ml. of hydrogen (theory 41 ml.) at 21° and 756 mm. in presence of Raney nickel: 0·44 g. of acid, m.p. 60·5–62° was obtained. Twice crystallized from light petroleum-ethyl acetate it melted at 63° alone or when mixed with the dihydro acid from synthetic *dl*-hydnocarpic acid.

The amide crystallized from ethanol in long prisms of m.p. 114·5–115·5° (alone or when mixed with the amide from synthetic dihydrohydnocarpic acid. (Found: N, 5·8%. Calc. for $C_{16}H_{31}ON$: N, 5·5%.)

SUMMARY

1. *dl*-Hydnocarpic acid has been synthesized from *cyclo*pent-2-enyl chloride by the Robinson long-chain synthesis (Robinson & Robinson, 1925), a method similar to that used by Perkins & Cruz (1927*b*) for chaulmoogric acid.

2. The accepted structure of hydnocarpic acid is confirmed.

We are indebted to Dr J. L. Simonsen, F.R.S., Director of Research, Colonial Products Research Council, Imperial Institute, for a supply of the oil of *Hydnocarpus wightiana*; and to Mr T. M. Sharp, of the Wellcome Research Institution, for a specimen of *d*-hydnocarpic acid.

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1948

The Absorption of Ammonia from the Rumen of the Sheep

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The peculiar anatomical arrangement of the digestive tract of the ruminant presents special problems in the elucidation of the processes of digestion in these species. The food is received, after swallowing, into the capacious rumen and reticulum where extensive fermentative changes are effected by the abundant micro-organisms which inhabit these organs. This unique arrangement confers on the ruminants an efficient mechanism for the digestion of cellulose and other polysaccharides of their exclusively herbaceous diet. The favourable conditions maintained in the rumen and reticulum for the growth of micro-organisms result in extensive conversion of the nitrogen of the animal's food to the use of the micro-organisms for their own growth. That ammonia should appear in the ruminal contents is not surprising in view of the fact that ammonia is the chief nitrogenous end product in the breakdown of proteins by bacteria, while at the same time ammonia is the chief source of nitrogen in the nutrition of many bacteria (Stephenson, 1939).

Schwarz & Steinlechner (1925) were unable to detect ammonia in the rumen contents of cattle, but their observation is at variance with that of all subsequent workers, e.g. Mangold & Schmitt-Kramer (1927); Harris, Work & Henke (1943); Wegner, Booth, Bohstedt & Hart (1941); Lenkeit & Becker (1938). Interest in the non-protein nitrogen fractions in the rumen contents has chiefly been directed to the phenomenon of the utilization by ruminants of urea and amides as a source of protein through the mediation of the micro-organisms of the fore stomachs; recent reviews of the extensive literature on this subject have been given by Smith (1945) and McClymont (1946). That urea is rapidly converted to ammonia by microbial activity in the rumen was demonstrated by Lenkeit & Becker (1938) and confirmed by later workers. However, it appears to have been overlooked that the saliva in those species studied contains urea in concentrations proportional to that in the blood (Hench & Aldrich, 1922, 1923; Schmitz, 1923; Komarov & Stávraky, 1940). Observations recorded in this paper show that urea occurs in the saliva of the sheep, and in view of the fact that the sheep secretes very large volumes of saliva (Scheunert & Trautmann, 1921; Scheunert & Krzywanek, 1930) this source of urea, and hence of ammonia, in the rumen contents assumes some significance.

Little attention has previously been paid to the role of ammonia in the digestion of natural foodstuffs in the rumen; some representative data on the changes in the concentration of ammonia in the rumen contents are presented in this paper. Ammonia in the ruminal contents appears to derive chiefly from the deamination of food proteins and from urea secreted in the saliva. Ammonia could disappear from the rumen liquor by its utilization by bacteria for their growth, by passage in the ruminal ingesta from the rumen to the abomasum, or by direct absorption.

Barcroft, McAnally & Phillipson (1944) demonstrated that volatile acids (acetic, propionic and butyric) are absorbed from the rumen. Further, it has long been known that ammonia is absorbed from the intestinal tract of monogastric animals (Folin & Denis, 1912; Parnas & Klisiecki, 1926; Koprowski & Uninski, 1939). These facts suggested that ammonia might be absorbed from the rumen of the sheep; the experiments reported in this paper demonstrate that this is indeed the case.

METHODS

Ammonia in blood was estimated by the method of Conway & Cooke (1939). For the collection of blood from the visceral veins, the technique developed by Barcroft *et al.* (1944) was used. The experimental animal was anaesthetized with nembutal (pentobarbital), the abdomen was opened and the visceral veins exposed. Blood was withdrawn into a syringe, with as little interference to blood flow as possible. No technique is as yet available for withdrawing blood from the ruminal veins in the normal, unanaesthetized animal.

Ammonia in rumen liquor was estimated by the method of Conway (1947) using potassium metaborate as the alkaline reagent. Corrections for the slight deamination caused by the alkali were applied. The rumen liquor was prepared by passing through muslin ruminal contents collected through a permanent rumen fistula.

Urea in saliva was estimated by the method of Conway & O'Malley (1942).

RESULTS

Ammonia in blood

Preliminary analyses of the jugular blood of normal sheep confirmed the general conclusion of Conway (1947) that in the blood of the general circulation there is almost no ammonia present. Conway has shown that ammonia is formed in human blood within a few minutes after collection, and that during absorption of ammonia in the Conway unit gradual deamination occurs. In the experiments quoted below the technique adopted necessitated the passage of some 2–4 min. from the time of insertion of the hypodermic needle into the vein until the blood sample had been delivered into the Conway units (analyses were performed in triplicate or quadruplicate). No data are available for applying corrections for the rate of ammonia formation or rate of deamination by alkali in the blood of sheep. For this reason Conway's corrections for deamination as applied to human blood have been used, but no correction has been applied for ammonia formation during the interval between collection of the blood and its mixture with the potassium carbonate in the Conway unit. Thus no special accuracy can be claimed for the values given for ammonia in blood drawn from the jugular vein or the carotid artery; it can only be concluded that these values are extremely small. In analyses of blood from the ruminal veins, the errors involved are of comparatively little significance.

The results of six experiments are given in Table 1; it was consistently found that the blood of the ruminal veins contained significant amounts of ammonia; the values averaged 1·7 mg. ammonia N/100 ml. blood.

Barcroft *et al.* (1944) had previously observed that the concentration of volatile fatty acids was higher in the anterior ruminal vein than in the posterior ruminal vein; they ascribed this to the fact that the posterior ruminal vein drained an area of the rumen which covered a gas space, which thus, in part, removed the ruminal mucosa from contact with the ingesta. In Exps. 5 and 6 (Table 1), analyses were made of blood from both ruminal veins and in each case the level of ammonia was slightly higher in the anterior ruminal vein.

The observations made in Exp. 2 confirm the findings of Folin & Denis (1912) and of Parnas & Klisiecki (1926) that ammonia is also absorbed from other regions of the gastro-intestinal tract.

In order to establish that the ammonia found in the ruminal veins is in fact derived from the ruminal ingesta, two experiments were performed. Two sheep were fasted for 24 hr. and then the contents of the rumen and reticulum were removed through a rumen fistula, and the rumen was washed out as thoroughly as possible with warm water. Under nembutal anaesthesia the oesophagus was ligated, the fluid again removed from the rumen and replaced with warm water, and the posterior ruminal vein exposed by laparotomy. Samples of blood from the ruminal vein were taken for estimation of ammonia. The fluid was then removed from the rumen and replaced with 4 l. of a warm solution of ammonium acetate. After an interval of 30 min., blood samples were again taken from the posterior ruminal vein and from the carotid artery. The results of the two experiments are set out in Table 2.

It has been found difficult to wash the rumen quite free of ingesta, and this explains why some ammonia remains in the rumen fluid after washing, and why the blood draining the washed rumen still contains more ammonia than the blood in the general circulation. The marked rise in the level of ammonia in the ruminal blood, after the rumen has been filled with a dilute solution of ammonium acetate, leaves no doubt that ammonia can be absorbed from the rumen.

Table 1. *Concentration of ammonia in the blood from different vessels in sheep*

Vein	Ammonia N (mg./100 ml. blood)					
Exps. ...	1	2	3	4	5	6
Jugular	0·0	0·0	0·1	—	—	—
Carotid (artery)	—	—	—	0·07	0·02	0·06
Posterior ruminal	1·9	1·9	1·5	1·4	2·2	1·3
Anterior ruminal	—	—	—	—	2·4	1·6
Abomasal	—	0·1	—	—	—	—
Intestinal	—	0·6	—	—	—	—
Caecal	—	0·8	—	—	—	—

Table 2. *Absorption of ammonia from the rumen of sheep*

	Ammonia N			
	Experiment 7		Experiment 8	
Origin of samples	(mg./100 ml. blood)	(mg./100 ml. ruminal fluid)	(mg./100 ml. blood)	(mg./100 ml. ruminal fluid)
Ruminal fluid, beginning of experiment	—	0·2	—	0·4
Posterior ruminal vein, 1st sample	0·13	—	0·36	—
Ruminal fluid containing ammonium acetate	—	15·4	—	22·6
Posterior ruminal vein, 2nd sample	0·93	—	1·64	—
Carotid artery	0·07	—	0·07	—

Ammonia in the rumen contents

In the course of studies on the digestion of protein in the sheep, numerous analyses of the content of ammonia in the rumen liquor were made; it has been repeatedly observed that ammonia N may constitute a significant fraction of the total N in the rumen liquor. A typical curve showing changes in the concentration of ammonia in the rumen liquor after feeding on pasture are given in Fig. 1; the animal was fasted overnight and returned to the pasture after two preliminary samples of rumen liquor had been collected. The figure shows clearly that the concentration of ammonia rose steadily for some hours after feeding on pasture, and attained a maximum value of about 35 mg. ammonia N/100 ml. rumen liquor.

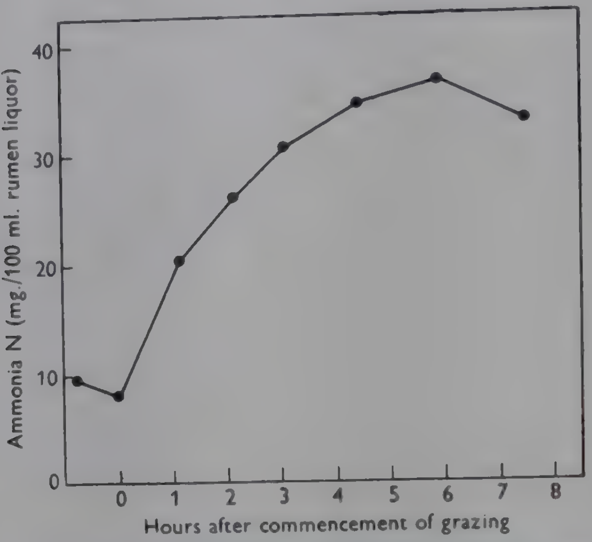

Fig. 1. Change in concentration of ammonia in the ruminal contents of a sheep after grazing at pasture.

Urea in saliva

Samples of mixed saliva were collected from three sheep on 2 days just prior to feeding. The values obtained for the concentration of urea in the saliva are given in Table 3.

Table 3. *Concentration of urea* N *in the saliva of sheep*

Sheep no.	Urea N on 2 days (mg./100 ml.)
1	8·2, 13·6
2	6·5, 7·4
3	15·0, 10·4

DISCUSSION

The fact that ammonia is absorbed from the rumen raises several interesting points. Since urea is present in the saliva it appears that a N circulation occurs during the normal digestive process; the salivary urea is converted by microbial activity in the rumen into ammonia, which may be absorbed into the venous blood and passed via the portal vein into the liver; here the ammonia will be converted into urea and again become available for secretion in the saliva. The significance of this cycle will clearly be dependent on the relation between the amount of urea N secreted daily in the saliva and the amount of ammonia N absorbed daily, for it seems probable that any nitrogen thus absorbed in excess of that secreted in the saliva would constitute a wastage in metabolism. Data are not yet available to allow accurate assessment of these quantities, but preliminary calculations suggest that ammonia N absorbed may well exceed by significant amounts the urea N secreted in the saliva. Barcroft *et al.* (1944) estimated the rate of flow of blood from the rumen and reticulum as 225 ± 65 ml./min. (This figure has been recalculated from the data of Barcroft *et al.* (1944) as the actual values for *total* blood flow from the rumen and reticulum are not quoted in their paper.) If then the efferent blood contains 1·5 mg. ammonia N/100 ml. and the rate of flow be taken as 200 ml./min., the absorption of ammonia amounts to 4·3 g. N/day. For comparison, values for saliva may be taken as 5 l./day with 10 mg. urea N/100 ml. representing a salivary secretion of 0·5 g. N/day. Further, it appears that nitrogen, derived from the food, may in part be lost to the animal by this means and will thus constitute a factor in determining the biological value of the food protein. It is evident, too, that when urea is fed to the animal as a source of nitrogen, part at least will be absorbed from the fore stomachs as ammonia and hence be valueless to the animal.

SUMMARY

1. Ammonia occurs only in traces (if at all) in the blood of the general circulation of the sheep while the venous blood draining the rumen contains about 1·5 mg. ammonia N/100 ml.; it is, therefore, concluded that ammonia is absorbed from the rumen. Addition of a solution of ammonium acetate to the rumen after removal of the ingesta produces a prompt rise in the level of ammonia in the blood draining the organ. It is calculated that the quantity of ammonia N absorbed from the rumen may be of the order of 4–5 g./day.

2. Urea occurs in significant quantities in the saliva of the sheep. Attention is drawn to the circulation of nitrogen in the normal digestive process, viz. urea in saliva is converted in the rumen into ammonia, which may be absorbed into the portal blood stream and converted by the liver to urea, which again becomes available for secretion in the saliva.

3. Ammonia derived from the deamination of proteins in the feed contributes an important fraction of the nitrogen of the ruminal ingesta.

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The Biochemistry of Bacterial Toxins

2. THE ENZYMIC SPECIFICITY OF *CLOSTRIDIUM WELCHII* LECITHINASE

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The general properties of a lecithinase present in the toxic culture filtrates of *Clostridium welchii* were described by Macfarlane & Knight (1941), who concluded that this enzyme was probably identical with *Cl. welchii* alpha toxin, the main lethal component of these filtrates. Since none of the *Cl. welchii* toxins examined hydrolyzed diphenyl-, monophenyl- or β-glycero-phosphate, or nucleic acid, this enzyme appeared to be a true lecithinase, as distinct from a phosphodiesterase, although the linkage attacked is of the diester type leading to the formation of phosphorylcholine and a diglyceride. In a further study of the enzyme specificity, Macfarlane (1942) reported briefly that kephalin was not decomposed by *Cl. welchii* toxin, but sphingomyelin was slowly hydrolyzed; the products of this hydrolysis, however, were not isolated.

Recently Zamecnik, Brewster & Lipmann (1947), who were unaware of this finding, examined the specificity of the toxin by an elegant adaptation of the manometric technique, and stated that it was inactive towards sphingomyelin, as well as to the other phospholipins tested. It seemed possible that the negative result of these authors with sphingomyelin was due to the short reaction time and low toxin concentration employed, but it was desirable to confirm the earlier positive results in more detail, since the specificity of the lipolytic activity of the toxin is a matter of some interest. *Cl. welchii* toxins in general appear to be free from phosphoesterases, lipase and nuclease, and the specificity of action might therefore be exploited for the differential estimation of phospholipins, for the elucidation of phospholipin structure or for the stepwise degradation of lipoprotein compounds.

The sample of sphingomyelin used in these experiments was kindly given to me by Dr O. Rosenheim, as one which, from its method of preparation as an ether-insoluble fraction, was free from lecithin; it contained, however, about 25 % cerebroside. Several hydrolyses of this crude sphingomyelin with *Cl. welchii* toxin have been carried out, in which from 70 to 90 % of the phosphorus present was converted into a water-soluble compound, with a satisfactory recovery of phosphorylcholine. It proved difficult, however, owing to the overlapping solubilities in the solvents tried, to separate the fatty product, which on the classical formula of sphingomyelin should be lignocerylsphingosine, from the accompanying cerebroside in sufficient yield and purity to characterize the product, though evidence of the formation of a nitrogenous product free from phosphorus and carbohydrate was obtained. It is clear also, since the starting material may be a mixture of 'sphingomyelins', that the hydrolysis product may be a mixture containing a variety of fatty acid residues, quite apart from any cerebroside impurity in the crude lipin.

Dr Lipmann has since informed me that his early data (manometric) showed a very slow reaction between the toxin and sphingomyelin, which was attributed to contamination of the sphingomyelin with a little lecithin, but that using larger amounts of toxin and a longer reaction time he has confirmed the decomposition of sphingomyelin. It appears therefore that *Cl. welchii* toxins have a curious specificity, which is presumably due to a single enzyme, in splitting off phosphorylcholine from lecithin and sphingomyelin, though neither kephalin (phosphatidylethanolamine or phosphatidylserine) nor, according to Zamecnik *et al.* (1947), glycerophosphorylcholine nor lysolecithin are attacked.

EXPERIMENTAL

Methods. The methods used for testing the enzymic activity of the toxin were similar to those previously described (Macfarlane & Knight, 1941), the reactions being followed by estimation of the acid-soluble organic P formed. One enzyme (lecithinase) unit (e.u.) = the amount producing 0·1 mg. P from excess lecithin at pH 7·6 and 37° in 15 min. under standard conditions.

Toxin. Glycerinated or dried toxin derived from *Cl. welchii*, type A, strain S 107, was used for the study of reaction rates, and a highly purified dry preparation, 354 H, for which I am indebted to Dr van Heyningen and Miss Bidwell, for the isolation experiments.

Substrates. The crude sphingomyelin used contained 2·7 % P, 2·5 % N, and 5·0 % galactose by orcinol estimation; calculated from this, the sample contained approximately 71 % of sphingomyelin and 23 % of cerebroside.

'Lecithin' was prepared from egg yolk, by repeated precipitations of the portion soluble in ether at 0° with acetone. The product usually contained 80–90 % lecithin.

A mixed preparation of 'lecithin-kephalin' was obtained from acetone-dried sheep brain by extraction with ether and repeated precipitation with acetone of the ether-soluble portion.

A purer preparation of 'kephalin' was obtained by repeated precipitation of the ethereal extract with 4 vol. ethanol.

Hydrolysis of sphingomyelin

Rate of hydrolysis. A representative protocol showing the comparative rate of hydrolysis of sphingomyelin and lecithin is shown in Table 1. At the same concentration, the hydrolysis of sphingomyelin is considerably slower than that of lecithin, but proceeded to the extent of 70 % in the conditions used. The hydrolysis, like that of lecithin, is activated by Ca ions and inhibited by NaF.

Isolation of products: Procedure (*a*). Crude lipin (0·69 g.) was emulsified in 25 ml. 0·02 M-$CaCl_2$, the pH adjusted to 8·4 with NaOH, and 10 mg. (600 e.u.) *Cl. welchii* toxin 354 H added in three portions at intervals of 3 hr. The mixture was incubated at 37°, being titrated back to pH 7·4 at intervals with 0·05 N-NaOH and finally to pH 8·4. The hydrolysis was allowed to proceed for 22 hr. in all, at which point 86 % of the P was water-soluble.

The aqueous portion was separated by filtration, evaporated to a small bulk on a water bath, refiltered, evaporated to dryness and re-extracted into 2 ml. water from which the crystalline Ca compound of phosphorylcholine was obtained on addition of ethanol. (Found: P, 9·1; N, 3·7; Cl, 10·1 %. Calc. for $C_5H_{13}O_4NPClCa.4H_2O$: P, 9·46; N, 4·25; Cl, 10·75 %.) The identity was further confirmed by determination of the hydrolysis constant in N-HCl at 100°, and by the rapid and complete hydrolysis of this phosphoric ester by bone phosphatase (phosphomonoesterase) to orthophosphate with liberation of choline.

The water-insoluble portion was extracted with cold acetone, dried and re-extracted with acetone. The total acetone-soluble material amounted only to 40 mg.; assuming that this material was a diglyceride derived from lecithin, the maximum amount of lecithin possibly present as an impurity in the crude sphingomyelin corresponded to less than 10 % of the organic P present. The acetone-insoluble material was fractionated by deposition from ethanolic solutions at different temperatures, the course of fractionation being followed by the P, N and carbohydrate content, as shown in Table 2.

Isolation of products: Procedure (*b*). Another hydrolysis, using 0·5 g. crude sphingomyelin, was carried out in a similar way, but the water- and acetone-insoluble material was extracted with cold chloroform. The fractions soluble and insoluble in chloroform, redeposited from ethanolic

Table 1. *Comparative hydrolysis of lecithin and sphingomyelin at 37°*

(Reaction mixtures contained 1·0 ml. aqueous emulsion of phospholipin (100 μg. P), 1 ml. borate buffer (Palitzsch), pH 7·6, and the additions below in a total volume of 6·0 ml.)

Cl. welchii toxin (Enzyme units)	Phospholipin	Additions	Time of incubation (min.)	P hydrolyzed (μg.)
1·1	Sphingomyelin	Nil	30	15
1·1	Sphingomyelin	$CaCl_2$	30	20
1·1	Sphingomyelin	$CaCl_2$	180	37
1·1	Sphingomyelin	NaF	180	12
1·1	Lecithin	Nil	30	30
1·1	Lecithin	$CaCl_2$	30	51
1·1	Lecithin	$CaCl_2$	180	76
1·1	Lecithin	NaF	180	21
3·3	Sphingomyelin	$CaCl_2$	180	71
3·3	Sphingomyelin	NaF	180	19

Table 2. *Balance sheet of the hydrolysis of crude sphingomyelin by* Cl. welchii *toxin*

	Quantities (mg.)			Composition (%)				
	Wt.	P	N	P	N	Carbohydrate (as galactose)	C	H
Original material	579	15·6	14·5	2·7	2·5	5·0	—	—
Calculated sphingomyelin (from P)	410	15·6	14·1	3·79	3·42	Nil	—	—
Calculated phrenosin (from galactose)	133	—	2·2	Nil	1·70	21·7	69·6	11·2
Calculated yield lignoceryl sphingosine	319	—	7·0	Nil	2·21	Nil	77·4	12·7
Found								
(1) P equivalent of acid formed	—	12·8	—	—	—	—	—	—
(2) Water-soluble portion	—	13·5	6·5	—	—	—	—	—
Cryst. phosphoryl choline	110	10·6	4·1	—	—	—	—	—
(3) Water-insoluble portion:								
Fraction from ethanol at 20°	86	1·65	1·46	1·92	1·7	9·2	—	—
Fraction from 95% ethanol at 20°	138	0	2·88	0	2·1	1·7	—	—
Fraction from ethanol at 0°	129	0·21	2·45	0·16	1·9	12·0	—	—
Mother liquors	83	0	—	0	—	4·0	—	—
Semi-crystalline material from pooled carbohydrate-low fractions from four hydrolyses	250	—	—	0	1·9	3·0	74·9	11·9

Table 3. *Hydrolysis of monoaminomonophosphatides by* Cl. welchii *toxin*

(Reaction mixture (6·0 ml.): 0·48 mg. lipin P; 3·0 e.u. toxin; 0·002 M-$CaCl_2$; pH 7·6, at 37°.)

Phospholipin	Amino N / Total N (%)	α-Amino N / Total N (%)	Calc. % of P as lecithin	% hydrolysis of P			
				1 hr.	2 hr.	3 hr.	4 hr.
(1) Ether-soluble acetone-insoluble (egg yolk)	22	Nil	78	70	74	78	78
(2) Ether-soluble acetone-insoluble (sheep brain)	83	23	17	17	19	22	22
(3) Ether-soluble ethanol-insoluble (sheep brain)	95	25	5	0	<5	<5	—

solution at 0°, contained 5·5 and 14·5% carbohydrate respectively, but repetition of this step on the carbohydrate-low fraction did not effect a further separation. This material was then fractionated by deposition from hot ethanol; the main fraction, which separated between 22° and 0°, contained P, 0·07; N, 1·9; carbohydrate, 4·5%.

Four hydrolyses of 0·5–0·6 g. crude sphingomyelin were carried out in all, in which from 67 to 91% of the lipin P was converted to a water-soluble form, with a satisfactory recovery of phosphorylcholine, while approx. 30–40% of the calculated sphingosine N of the phospholipin was recovered in a P-free carbohydrate-low fraction containing 1·9–2·1% N. This pooled material was insoluble in cold ethanol, acetone or ether, dissolving readily on warming in ethanol or acetone and redepositing on cooling. After several fractionations, the deposit from 95% aqueous ethanol was a semi-crystalline mass of irregular plates but still gave a reaction on heating with orcinol and H_2SO_4 which indicated the presence of 2–3% carbohydrate (Table 2). In view of the possibility that the starting material contained a mixture of 'sphingomyelins', as well as the cerebroside impurity, the further identification by degradation of this small amount of material was not attempted.

Action of Cl. welchii *toxin on kephalin*

The action of *Cl. welchii* toxin on individual monoaminomonophosphatides was determined by comparison of the maximal production of water-soluble P from ether-soluble phospholipins prepared from egg yolk and brain with the amino N and α-amino N of the sample. Table 3 shows that the maximum percentage hydrolysis of P corresponded with the percentage content of non-amino (lecithin) N, while practically no hydrolysis was observed in a 'kephalin' fraction from sheep brain containing 25% of the total N as α-amino N. The conclusion drawn from this result, that neither phosphatidylethanolamine nor phosphatidylserine was hydrolyzed by *Cl. welchii* lecithinase, has been confirmed by Zamecnik *et al.* (1947) using pure samples of these phospholipins.

SUMMARY

1. The action of *Cl. welchii* toxin on a sample of crude sphingomyelin has been examined. Approximately 90% of the lipin P was converted into a water-soluble form; the hydrolysis is slower than that of lecithin P in similar conditions, but like that of lecithin is activated by Ca ions and inhibited by NaF. Phosphorylcholine was recovered quantitatively from the hydrolysis products, together with a P-free product approximating to the composition of lignocerylsphingosine, which was not obtained pure.

2. The maximum hydrolysis of lipin P in mixed lecithin-kephalin preparations corresponded with the percentage content of non-amino (lecithin) N.

3. It is concluded, from the manner of hydrolysis, that *Cl. welchii* lecithinase hydrolyzes sphingomyelin, as well as lecithin, with production of phosphorylcholine, but does not attack phosphatidylethanolamine nor phosphatidylserine.

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The Biochemistry of Bacterial Toxins

3. THE IDENTIFICATION AND IMMUNOLOGICAL RELATIONS OF LECITHINASES PRESENT IN *CLOSTRIDIUM OEDEMATIENS* AND *CLOSTRIDIUM SORDELLII* TOXINS

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(*Received 29 October 1947*)

Nagler (1939) observed that when *Clostridium welchii* type A was grown in human serum an opalescence developed and eventually a layer of fat rose to the surface. Macfarlane, Oakley & Anderson (1941) found that *Cl. welchii* toxin acts on a clear emulsion of egg yolk to give a similar but more marked opalescence, and that this reaction is due entirely to the α-toxin, the main lethal component of *Cl. welchii* type A culture filtrates; this is termed the lecithovitellin (L.V.) test. Subsequently, *Cl. welchii* toxin was found to contain a lecithinase (Macfarlane & Knight, 1941) which is probably identical with the agent responsible for the Nagler and L.V. reaction, and therefore with the α-toxin.

Hayward (1941, 1943) and Crook (1942) found that other species of *Clostridium*, particularly *Cl. oedematiens*, and also some aerobic spore bearers, may give positive Nagler or L.V. tests; this fact, as pointed out by Hayward (1943), does not militate against the specificity of the test for the detection of *Cl. welchii*, as the essence of the test is not merely a positive reaction but the inhibition of such a reaction by the specific antitoxin. There is, however, a tendency to regard any bacterial agent giving a positive L.V. test as a 'lecithinase', which is clearly unwarrantable without chemical evidence and may lead to confusion on the nature of the bacterial agents.

Macfarlane (1942) briefly reported the identification of a lecithinase in *Cl. oedematiens* toxins, which was immunologically distinct from the main lethal component, and also from *Cl. welchii* lecithinase. Oakley, Warrack & Clarke (1947), in a comprehensive study of the antigenic components of *Cl. oedematiens* toxins, found that the L.V. factor (γ-toxin) in *Cl. oedematiens* type A filtrates was immunologically distinct from that (β-toxin) present in type B filtrates. Through the courtesy of Dr Oakley, I have been able to show that the immunological behaviour of the lecithinases present in each of these types was similar to that of the L.V. factor, thus indicating that *Cl. oedematiens* β- and γ-toxins are lecithinases. The identification of these lecithinases, and of a lecithinase present in culture filtrates of certain strains of *Cl. sordellii* or *Cl. bifermentans*, is described below.

EXPERIMENTAL

The general methods of procedure and analysis were similar to those used by Macfarlane & Knight (1941). Lecithinase activity was determined by estimation of acid-soluble P liberated from lecithin, and the L.V. factor by the development of turbidity in a clear emulsion of egg yolk.

Toxins and antitoxins

Cl. oedematiens. For the initial experiments in 1942, dry or glycerinated toxins derived from *Cl. oedematiens* 'Albiston' were used. This type B strain toxin was in routine use in the Serum Department of the Lister Institute at Elstree for the hyperimmunization of horses; the various antitoxic sera from Elstree examined for antilecithinase activity were B type (anti-β-toxin). *Cl. oedematiens* type B toxin, O.C. 91044, with corresponding antisera Todd 3 and Todd 61, and type A toxins, A.E. 327, and O.C. 19345 with antisera 202 and 645 were given by Dr Oakley.

Cl. sordellii (or *bifermentans*). Culture filtrates were prepared in this laboratory from five strains (referred to for convenience as *Cl. sordellii*) obtained from Miss Hayward, described as: A 127 A (*sordellii* or *bifermentans*) from necrotic wound; N.C.T.C. 2919, *sordellii*; A 90 *b*, *sordellii*, from a case of gas gangrene; A 112 (*sordellii* or *bifermentans*); 7851 *a* (*sordellii* or *bifermentans*) from a gangrenous wound.

The strains were subcultured on Robertson's meat medium, and inoculated into the peptone medium described by Macfarlane & Knight (1941). After growth at 37° for 4–18 hr., the fluid was cleared by centrifuging and tested immediately for enzyme activity. The lethality in mice was not tested. *Cl. sordellii* antitoxin was not available.

Expression of results

One lecithinase or enzyme unit (e.u.) is that amount producing 0·1 mg. acid-soluble P from lecithin in 15 min. in standard conditions (Macfarlane & Knight, 1941). One egg yolk unit (y.u.) is that amount producing the same opacity under standard conditions as a test dose of *Cl. welchii* toxin containing one e.u. The unit of *Cl. oedematiens* α-antitoxin (anti-lethal) is the International Unit, those for β- and γ-antitoxin are those defined by Oakley *et al.* (1947) with reference to selected sera.

The antilecithinase activity of *Cl. oedematiens* antitoxin sera is calculated on the basis that 0·04 unit causes 50% reduction in the hydrolysis under standard conditions by a test dose of *Cl. oedematiens* toxin containing 2 e.u. lecithinase. This was adopted because 0·04 unit of *Cl. welchii* antilecithinase (the Elstree Standard no. 1 *Cl. welchii* antitoxin having been arbitrarily selected by Macfarlane & Knight as a standard containing 95 units/ml.) causes approximately 50% reduction in the hydrolysis by 2 e.u. of *Cl. welchii* lecithinase.

These *Cl. welchii* and *Cl. oedematiens* antilecithinase units are therefore comparable in a biochemical sense; but since the inhibiting power of the antilecithinase will depend on the total amount of antigen (toxin or toxoid) present in a particular sample of toxin, the *Cl. oedematiens* antilecithinase unit is not a fixed standard against all toxins. As the *Cl. oedematiens* type A and type B lecithinases are probably identical with the γ- and β-toxins respectively, the antilecithinase activity should preferably be expressed in future in γ- and β-units by comparison of the inhibiting power with that of the standard sera against the same test dose of toxin.

Hydrolysis of lecithin by Cl. oedematiens *toxin*

Though the liberation of acid-soluble P from lecithin by *Cl. oedematiens* toxin can readily be demonstrated, the quantitative recovery of the hydrolyzed products was more difficult than in *Cl. welchii* hydrolysis, for the low lecithinase activity of the toxins involved either the addition of a large amount of impurities or the prolongation of the hydrolysis, with the risks of autolysis or secondary reactions by associated enzymes.

Identification of products of hydrolysis by type B toxin. The following protocol is representative of a number of hydrolyses.

Egg lecithin (0·5 g. containing 18 mg. P) was emulsified in 15 ml. 0·01 M-$CaCl_2$, the pH adjusted to 7·4 and 15 mg. *Cl. oedematiens* type B toxin (4·5 e.u.) added. The hydrolysis was carried out at 37°, more enzyme being added at 24 hr. and the pH adjusted to 7·4 with NaOH at intervals. After 45 hr., 0·56 mmol. acid had been liberated, equivalent to 95% hydrolysis. The mixture was then separated into water-soluble and ether-soluble fractions by shaking several times with ether.

The aqueous solution, which contained 91% of the original lipin P as organic P, was evaporated to dryness and the crystalline Ca component of phosphorylcholine obtained from the ethanolic extract of the residue. (Found: P, 9·32; N, 3·7; Cl, 11·4; H_2O, 21·6%. Calc. for $C_5H_{13}O_4NClPCa.4H_2O$: P, 9·46; N, 4·25; Cl, 10·75; H_2O, 21·86%.) The yield in the first crop was low, only 25% of that calculated from the organic P liberated; in subsequent hydrolyses the yield of crystalline material was better, up to 60%.

The ethereal solution was evaporated and the residue was extracted with acetone. The dry weight (0·310 g.) of acetone-soluble material was equivalent to an 85% recovery of a diglyceride, calculated as palmityloleylglyceride; it had an acid value 2·7 and saponification value 176 (calculated 179).

Hydrolysis by type A toxin. (1) A lecithin emulsion containing 21·0 mg. lipin P in 0·02 M-$CaCl_2$ was treated with 20 mg. type A toxin O.C. 19345 at pH 7·4 and 37°, a further 20 mg. enzyme being added after 4 and 20 hr. The hydrolysis was stopped after the liberation of one equivalent of acid and the fatty portion separated from the aqueous solution by extraction with ether. The ethereal solution was evaporated and the residue extracted with acetone, leaving some acetone-insoluble material. The acetone-soluble material (380 mg.), dissolved in ethanol, had a saponification value of 167 but an acid value of 20.

The aqueous solution contained only 15·9 mg. P (76% of the calculated amount) of which 14·1 mg., all as organic P, were recovered on treatment of the evaporated and filtered solution with $CaCl_2$ and ethanol. All the organic P in a test sample was rapidly hydrolyzed by bone phosphatase, indicating the presence of a monophosphoric ester. The material was redeposited, on addition of ethanol to the aqueous solution, in a more or less crystalline state similar to that of the crystalline Ca compound of phosphorylcholine, but the analysis indicated some impurity. (Found: N, 3·4; Cl, 7·2; P, 8·1%. Calc. for $C_5H_{13}O_4NClPCa.4H_2O$: N, 4·25; Cl, 10·75; P, 9·46%.)

(2) The results suggested that the type A toxin contained both a lecithinase splitting off phosphorylcholine, and a lipase attacking either lecithin or the diglyceride formed by the primary action of the lecithinase. A second hydrolysis was carried out on an emulsion containing 17 mg. lipin P, in similar conditions but with 50 mg. toxin initially. The hydrolysis was stopped after 47 hr., when 2·3 equivalents of acid had been liberated. The mixture was acidified with HCl and extracted with ether, the ethereal and aqueous solutions being washed with water and ether respectively.

The aqueous solution contained 13 mg. P (76% of the original lipin P), all in an organic form which was rapidly hydrolyzed by bone phosphatase. A semi-crystalline Ca compound was obtained on treatment of the concentrated solution with ethanol, which had a P : N ratio 1 : 0·9; the analysis again indicated the presence of some impurity.

The ethereal solution on evaporation gave 314 mg. fatty material, which in ethanolic solution required 4·95 ml. 0·1 N-alkali for neutralization, equivalent to an acid value of 88·5 or approximately 140 mg. free fatty acid as oleic or palmitic acid. The neutral fat and the soap were separated by extraction with ether, and the content of saturated and unsaturated acids in each determined approximately by one separation of the Pb salts in 95% ethanol.

The fatty acids (138 mg.) recovered from the soap gave 85 mg. insoluble Pb salt, equivalent to 60·6 mg. palmitic acid, while 70 mg. fatty acid were recovered from the soluble Pb salt.

The neutral fat (161 mg.) gave 144 mg. acetone-soluble fat, with an acid value less than 4, which was saponified in ethanolic KOH. The saponification value of 159 was approximately that of oleyl or palmityl monoglyceride (156–157); 106 mg. fatty acid were recovered yielding 56 mg. insoluble Pb salts (equivalent to 40 mg. palmitic acid), and 65 mg. fatty acid from the soluble Pb salts.

In both these hydrolyses, therefore, more than 70% of the lipin P was recovered as a monophosphoric ester with a P : N ratio of approximately unity, and there seems little doubt that *Cl. oedematiens* type A toxin contains a lecithinase similar in action to that of type B toxin and *Cl. welchii* toxin, which decomposes lecithin into phosphorylcholine and a diglyceride. Type A toxin contains in addition a lipase which can split off both saturated and unsaturated fatty acids, either from the diglyceride or possibly from lecithin itself. In the latter case the products of hydrolysis by this second enzyme alone might include a monofatty-acid derivative of glycerophosphorylcholine, as well as glycerophosphorylcholine. The ratio of all the products formed by the toxin would naturally be dependent on the relative activities of the two enzymes, but the difficulty experienced in isolating analytically pure phosphorylcholine suggests that, though the γ-toxin is predominant, traces of other primary products of lecithin were formed.

The identification of two lipolytic enzymes in *Cl. oedematiens* type A toxin—a lecithinase (γ-toxin) similar in action to *Cl. oedematiens* β-toxin and to *Cl. welchii* α-toxin, and a lipase splitting off fatty acids—is consistent with the morphological appearance of type A strains in plate tests and the identification of various components in the culture filtrates by immunological tests. Nagler (1945) showed that the surface of colonies of *Cl. oedematiens* type A, grown on egg-yolk blood agar, is covered with a 'pearly layer', while type B strains do not produce this effect. Oakley *et al.* (1947) distinguished in type A culture filtrates two components producing opalescence in egg yolk: the γ-toxin and a second factor probably responsible for the 'pearly layer', named ϵ-toxin. It seems reasonable to suppose that the action of the ϵ-toxin is that of the lipase forming a pearly layer of fatty acid upon the colony, but further work is required to identify this enzyme by the inhibitory action of anti-γ and anti-ϵ sera, and to characterize its biochemical nature.

Effect of pH, *activators, etc.* *Cl. oedematiens* lecithinase (β-toxin) is active over a wide pH range with a broad optimum from 6·0 to 7·6. The pH activity curve was not determined with great accuracy, as it differed somewhat in shape with different toxins, which were all crude.

The enzymic activity was in general increased by the addition of CaCl$_2$ to the reaction mixture and inhibited by addition of NaF. These crude toxins were, however, active in the absence of added Ca, probably owing to the presence of sufficient Ca in the toxin and lecithin.

Lecithinase content of various toxins. The enzymic activity/unit weight or unit volume was determined by estimation of acid-soluble organic P liberated by measured amounts of the toxin in the following conditions: 1 ml. borate buffer (Palitzsch), pH 7·1; 0·2 ml. 0·3 M-CaCl$_2$; 1 ml. 2·5% aqueous lecithin; toxin or water to 6·0 ml.; incubated at 37° for 15 min.; acidified with 1·0 ml. 10% trichloroacetic acid and filtered.

Table 1 shows the lecithinase activity of various toxic culture filtrates, or dry toxins prepared from these, together with the lethal capacity (LD$_{50}$) in mice. The lecithinase activity of *Cl. oedematiens* toxins was very much lower than that of *Cl. welchii* toxins, and varied independently of the main lethal component; *Cl. oedematiens* type A toxins appeared generally to be lower in activity than type B toxins.

Table 1. *Lecithinase activity* (*e.u.*) *and lethality* (*LD*$_{50}$) *of* Cl. welchii *and* Cl. oedematiens *toxins*

Toxin	Values/mg. dry or ml. fluid toxin		Ratio LD$_{50}$/e.u.
	e.u.	LD$_{50}$	
Dry toxins			
Cl. welchii, type A, W.E. 5	12·2	25	2
Cl. oedematiens, type A, A.E. 327	0·06	—	—
Cl. oedematiens, type A, A.E. 208	0·10	—	—
Cl. oedematiens, type A, O.C. 19345	0·15	—	—
Cl. oedematiens, type B, Oe I	0·12	200	1,670
Cl. oedematiens, type B, Oe II	0·30	—	—
Cl. oedematiens, type B, 12 XB	0·29	—	—
Fluid toxins			
Cl. welchii, type A, K. 178	210	400	1·9
Cl. welchii, type A, K. 188	294	450	1·5
Cl. oedematiens, type B, V.F.I	1·5	6,000	3,900
Cl. oedematiens, type B, K.I	1·0	12,000	12,250
Cl. oedematiens, type B, O.C. 91044	1·4	—	—

Antilecithinase activity of Cl. oedematiens *antitoxic sera*

The general method for measuring the inhibiting action of antitoxic sera was as follows: 1·0 ml. of toxin containing 2 e.u. was allowed to combine with two or three different amounts of antitoxic sera in a total volume of 3·8 ml. for 10 min. at room temperature; borate buffer, $CaCl_2$ and lecithin were added and the hydrolysis measured in the usual way. The percentage inhibition of the hydrolysis by the same test dose of toxin without antitoxin in the same test was plotted against the amount of antitoxin, the amount equivalent to 50% inhibition being regarded as containing 0·04 antilecithinase units. Using homologous antitoxins which produced complete inhibition in excess, an approximately linear relationship obtained in the above conditions.

Table 2. *Antilecithinase titres of* Cl. oedematiens *type A and type B antisera against type B and type A lecithinase*

(1 e.u. of toxin A.E. 327 = 17 mg. ≡ 8 test doses for L.V. test. 1 e.u. of toxin O.C. 91044 = 0·7 ml. ≡ 31 test doses for L.V. test.)

	Antilecithinase units/ml. serum against 1 e.u. test toxin		Antitoxin titres (L.V. units/ml.)	
Serum	Type A toxin A.E. 327	Type B toxin O.C. 91044	γ-units	β-units
Type A 202	5·0	<0·2	700	0·5
644	7·7	—	700	2·0
Type B Todd 3	0·4	40	120	2500
Todd 61	0·2	—	65	450

A number of type B antitoxic horse sera prepared by routine immunization, and containing from 750 to 2500 international units/ml., were tested against the type B toxin Oe II and found to contain small amounts of antilecithinase (0·2–3·0 units/ml.). The ratio i.u./antilecithinase units varied from 250 to 10,000; thus the antilecithinase is clearly distinct from the anti-lethal toxin (Oakley's α-antitoxin).

Immunological specificity of type A and type B antilecithinase. The cross reactions between type A and type B antitoxic sera and type B and type A lecithinases were tested in a similar manner, but with a lower test dose of toxin (1 e.u.) owing to the small amounts available. The approximate antilecithinase titres against the same number of e.u.'s of the homologous and heterologous lecithinases are shown in Table 2, together with the titres for β- and γ-antitoxin determined by Dr Oakley. There is clearly a marked difference between the inhibiting power against the homologous and the heterologous enzyme. Considering the limits of accuracy of the tests, and the different basis of testing, there is also a substantial agreement between the ratio of the antilecithinase titre of a particular serum against the homologous and the heterologous toxin, and the ratio of its β- and γ-antitoxin content. It seems fair to conclude that the type A and type B lecithinases are identical respectively with the γ- and β-toxins distinguished by Oakley *et al.* (1947).

A protocol (Table 3) shows that, while there was practically no inhibition of the β-lecithinase by the type A serum, there was complete inhibition of the γ-lecithinase by the type B serum (Todd 3) if sufficient serum was added. Inspection of the titres for various sera given by Oakley *et al.* (1947, Table 8) shows that the ratio γ/β-units in a β-rich serum was always considerably higher than the ratio β/γ-units in a γ-rich serum. This is probably not a genuine cross reaction between β-antitoxin and γ-lecithinase, but is due simply to the fact that all the horses had received a preparatory dose of alum-precipitated toxoid from a type A (γ-toxin) filtrate; it is at least unlikely that complete inhibition in the lecithinase test, which in contrast to the egg-yolk test is augmented rather than inhibited by non-specific protein, could be due to non-specific inhibition by a low-titre serum.

Table 3. *Inhibition of type A lecithinase by type B antisera*

Toxin	Antiserum (ml.)	Hydrolysis (% of test dose)
Type A		
Test dose 1 e.u. γ-lecithinase	Type A 0·004	68
	0·01	32
	0·02	10
	Type B 0·007	96
	0·033	79
	0·10	49
	0·20	16
Type B		
Test dose 1 e.u. β-lecithinase	Type A 0·004	94
	0·02	93
	Type B 0·001	55
	0·007	0
	0·02	0

Species specificity of Cl. welchii *and* Cl. oedematiens *lecithinase.* The cross reactions of *Cl. welchii* type A and *Cl. oedematiens* type B antitoxins with the heterologous enzymes were examined in a similar way. There was practically no inhibition of either of the enzymes by the heterologous antitoxin, in amounts greatly exceeding those inhibiting the homologous enzyme.

Lecithinase activity of Cl. sordellii *culture filtrates*

Egg-yolk and lecithinase tests. Filtrates of 23 hr. cultures of five strains of *Cl. sordellii* were tested for their capacity to produce turbidity in a clear emulsion of egg yolk, in comparison with a test dose (1 e.u.) of *Cl. welchii* toxin. Three of the strains gave a positive reaction (N.C.T.C. 2919, A 90 B, A 112) with values 1·0, 0·9 and 0·5 y.u./ml. The first two filtrates were tested for lecithinase by estimation of acid-soluble P liberated from lecithin, and gave values of 0·7 e.u./ml. In various tests on culture filtrates from these strains, the lecithinase activity was lower, to a variable extent, than the egg-yolk value, indicating the presence of a second factor affecting egg yolk. As the routine test for lecithinase was complicated by the presence of inorganic P in a culture filtrate of low activity, an attempt was made with one filtrate to purify the enzyme by salting it out, but the dialyzed solution showed an even greater discrepancy between the egg factor (3 y.u./ml.) and the lecithinase (0·9 e.u./ml.).

Decomposition of egg yolk. The decomposition of egg yolk by a *Cl. sordellii* filtrate and by *Cl. welchii* toxin was followed with reference both to the conversion of lipin P to an acid-soluble form, and to the liberation of acid-soluble N. Table 4 shows that almost all the egg-yolk P, including presumably the bound lipin P as well as the ether-soluble P, was converted into acid-soluble organic P; there was slightly more formation of acid-soluble N by *Cl. sordellii* than by *Cl. welchii* toxin. It is possible that *Cl. sordellii* cultures contain an enzyme attacking the yolk protein and thereby affecting the turbidity development in the egg-yolk test, but it seems more probable that this toxin, like the *Cl. oedematiens* type A toxin, contains a lipase, for *Cl. sordellii* also gives a 'pearly layer' in plate tests. The products of hydrolysis of lecithin by *Cl. sordellii* toxin were not identified, but the action of the lecithinase is apparently similar to that of *Cl. welchii* in so far as an acid-soluble phosphoric ester is produced.

Inhibition by heterologous antitoxic sera. Owing to their low lecithinase activity, the culture filtrates from these strains of *Cl. sordellii* were not suitable for tests of the antilecithinase activity of sera, but some tests were carried out with the egg-yolk test. With one filtrate, inhibition either by *Cl. welchii* or *Cl. oedematiens* antitoxic sera was maximal at 50 %; with other filtrates complete inhibition was sometimes observed. Further work is required on purified and concentrated preparations of *Cl. sordellii* lecithinase to determine whether this inhibition is a true partial inhibition of the lecithinase, or a specific inhibition of a second factor affecting egg yolk, or a non-specific inhibition of the development of turbidity by the excess protein added. So far as it goes, the result is in accord with Hayward's (1943) finding in plate tests, that the Nagler reaction of some strains of *Cl. bifermentans* or *Cl. sordellii* is completely inhibited, and that of other strains partially inhibited, by *Cl. welchii* antitoxin.

DISCUSSION

It has been shown previously that the lecithinase present in *Cl. welchii* toxin is probably identical with *Cl. welchii* α-toxin, the main lethal, necrotic and haemolytic component of *Cl. welchii* type A toxins. Evidence has now been presented that culture filtrates of *Cl. oedematiens* contain a lecithinase similar in biochemical action to *Cl. welchii* lecithinase, but quite distinct from the main lethal component. The lecithinases present in culture filtrates from *Cl. oedematiens* type A and type B strains are immunologically distinct from each other, and there seems little doubt that they are identical respectively with the γ- and β-toxins described by Oakley *et al.* (1947). These authors point out that, as lecithinases, β- and γ-toxins might be expected to be haemolytic, necrotic and lethal, and that their own failure to demonstrate a lethal effect with certainty, after adsorption of the main lethal component with an appropriate antitoxic serum, was probably due to the small amount of lecithinase present. Assay of the lecithinase activity of various *Cl. oedematiens*

Table 4. *Liberation of* P *and* N *from egg yolk by* Cl. welchii *and* Cl. sordellii *toxins*

(Reaction mixture: 10 ml. yolk emulsion (1·1 mg. total P) and 2 ml. toxin (2 e.u.) or saline; 4 hr. at 37°.)

	Found after incubation (mg.)		
Determination	Control, without toxin	With *Cl. welchii* toxin	With *Cl. sordellii* toxin
P in ether-soluble fraction	0·56	Nil	Nil
Ortho P in trichloroacetic acid-soluble fraction	Nil	Nil	Nil
Total P in trichloroacetic acid-soluble fraction	Nil	0·96	0·97
N in trichloroacetic acid-soluble fraction	Nil	0·90	1·35

filtrates shows that this explanation is probably correct, since the maximum content found (1·5 e.u./ml.) is only equivalent (by comparison with *Cl. welchii* lecithinase) to approximately 3 LD_{50} doses/ml. for the mouse, compared with 6000–1200 LD_{50} doses/ml. of the main lethal component (*Cl. oedematiens* α-toxin).

The immunological and enzymic specificities of the lecithinases present in *Cl. welchii* and *Cl. oedematiens* toxins offer some entertaining facts for those interested in the relationship between chemical configuration and biological action. It may be supposed that the group responsible for lecithinase activity is closely similar in the three enzymes (*Cl. welchii* α-toxin, *Cl. oedematiens* β- and γ-toxins); the fine configuration of *Cl. welchii* lecithinase is such that it decomposes lecithin and, probably but less readily, sphingomyelin, but not kephalin (Macfarlane, 1942, 1948) while the failure to attack lysolecithin and glycerophosphorylcholine (Zamecnik, Brewster & Lipmann, 1947) suggests that the presence of two fatty acids is essential for orientation of the substrate molecule. Each of these enzymes is immunologically distinct; since the antilecithinase groups of the specific antibodies are presumably as similar to each other as the lecithinase groups of the antigens, it may be inferred that the immunological distinction between these antibodies is conferred by a 'species' or 'type' group in the antigen. But in other instances the immunological distinction is presumably conferred by the enzymic or 'toxic' group of the antigen, e.g. the distinction between the antilecithinase, antihyaluronidase, anticollagenase, etc. of *Cl. welchii* antitoxic sera. Thus these bacterial enzymes carry in effect two specific antigenic configurations, e.g. '*W* + lecithinase' or 'Oe A + lecithinase'; whether these configurations are discrete or whether they are interlocked to form a unique configuration, the structure is such that it can combine specifically both with the substrate of the enzyme, and with a type-specific anti-enzyme.

The immunological specificity of these 'toxic' enzymes is no doubt a reflexion of the stereochemical obtrusiveness of the antigenic structure, which is not without bearing on the virulence of the toxins. Discussing the behaviour of toxic enzymes, Macfarlane & Knight (1941) said, 'The toxin can primarily affect only those cells which contain the appropriate substrate for its enzymic activity, *and then only if this substrate is accessible to it*'. The very interesting fact has been pointed out by Oakley *et al.* (1947) that sheep red cells are readily attacked by the lecithinase *Cl. welchii* α-toxin, but are insensitive to the lecithinases *Cl. oedematiens* β- and γ-toxins; the latter haemolyze horse red cells, which are not attacked by *Cl. welchii* α-toxin. One possible explanation of this phenomenon is that stereochemically the specific blood-group substance of the host permits or hinders the approach of the type-specific group of the lecithinase, and thus permits or prevents the orientation of the enzyme towards the lecithin of the red-cell membrane. A similar kind of interference may well be the basis of the relative 'susceptibility' or 'resistance' of different host species to a particular toxin. It is clear that the activity of a bacterial enzyme towards a biological structure cannot be predicted merely on the basis that the structure contains the necessary substrate.

SUMMARY

1. *Cl. oedematiens* culture filtrates contain a lecithinase similar in biochemical properties to that present in *Cl. welchii* filtrates.

2. The lecithinases present in *Cl. oedematiens* type A and type B filtrates are immunologically distinct from each other, and from *Cl. welchii* lecithinase, in that their activity is inhibited by a homologous but not inhibited by a heterologous antitoxic serum. The type A and type B lecithinase are probably identical respectively with the γ- and β-toxins of *Cl. oedematiens* filtrates.

3. *Cl. oedematiens* type A toxins also contain a lipase, which is possibly identical with the factor (ε-toxin) responsible for the 'pearly layer' effect in colonies of this organism.

4. Culture filtrates of certain strains of *Cl. sordellii* also contain a lecithinase which is not yet characterized.

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1948

The Effect of Alcohols on the Hydrolysis of Sodium Phenolphthalein Diphosphate by Prostatic Extracts

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King (1943) synthesized several phosphoric esters which liberate chromogens after phosphatase hydrolysis. Among these were the calcium, barium and lead salts of phenolphthalein phosphate. He found, however, that these compounds were not suitable as a substrate for plasma phosphatase determinations owing to the slow rate of hydrolysis. Huggins & Talalay (1945) synthesized the sodium salt of phenolphthalein phosphate and considered it to be sufficiently rapidly hydrolyzed by phosphatase to constitute a suitable substrate for measuring phosphatase activity in plasma and other biological fluids. They described a method of measuring acid phosphatase activity using this substrate buffered at pH 5·4 and measuring colorimetrically the free phenolphthalein liberated. Whilst comparing this method with that of Gutman, Gutman & Robinson (1939), it was noticed that the addition of ethanol to the digest greatly increased the free phenolphthalein liberation.

EXPERIMENTAL

Preparation of enzyme. Human prostate was used as enzyme source. Partial purification was effected by the method of Kutscher & Worner (1936) slightly modified. The prostate was finely chopped, suspended in three times its own weight of distilled water, and autolyzed under toluene for 3 or 4 days. After centrifuging, the supernatant fluid was dialyzed against distilled water, and the protein precipitate filtered off. The preparation was freeze-dried and stored in the refrigerator. For use 6 mg. were dissolved in 50 ml. of distilled water.

Pentasodium phenolphthalein diphosphate was synthesized and acid-phosphatase activity estimated by the method of Huggins & Talalay (1945). Chloroform, however, was omitted from the substrate solution because irregular results were found in its presence.

Procedure. The phosphate liberated was measured as in the Bodansky (1933) method for the estimation of alkaline phosphatase. Simultaneous estimations of phenolphthalein and phosphate were carried out after digestion at 37° for 1 hr., both without any alcohol, and in the presence of ethanol, methanol, *n*-propyl and *iso*propyl alcohols and ethylene glycol.

The following buffered substrate was used: 1·216 g. sodium phenolphthalein phosphate, 11·7 g. sodium acetate (hydrated) and 0·79 ml. glacial acetic acid, dissolved in water and made up to 1 l. Each estimation was carried out in duplicate. Colorimeter tubes were set up in the water bath at 37° containing:

Tube (1) 2·5 ml. buffered substrate; 2·5 ml. distilled water; 0·5 ml. enzyme solution.

Tube (2) 2·5 ml. buffered substrate; 2·5 ml. diluted alcohol; 0·5 ml. enzyme solution.

1 ml. was immediately removed and discarded from (1), and 1 ml. from (2) was removed and run into a tube containing 5 ml. 12 % (w/v) trichloroacetic acid and 4 ml. distilled water. This was centrifuged, and the initial phosphate determined by the amount of colour developed from 5 ml. of supernatant fluid. The tubes were then incubated for 1 hr., after which sufficient pure alcohol was added to (1) to give it the same concentration of alcohol as (2). The volume of (2) was then adjusted to that of (1) by adding alcohol of the appropriate strength. This dilution of the digest was allowed for in calculating the results. 1 ml. was then removed from (1) and (2) into tubes containing 5 ml. 12 % (w/v) trichloroacetic acid and 4 ml. distilled water, centrifuged, and the supernatant fluid used to obtain the amount of free phosphate after digestion. To the remainder of the solution in the colorimeter tubes, glycine buffer and water were added, and the amount of free phenolphthalein liberated determined.

RESULTS

The effect of different concentrations of ethanol on the phenolphthalein liberated by the enzyme was determined. Approximately 15 % (v/v) was found to show the greatest effect (Fig. 1). For the other alcohols, a concentration giving a marked increase in the amount of phenolphthalein liberated was chosen. The differences between the amounts of phenolphthalein and phosphate liberated with and without alcohol were measured, and the percentage increase or decrease calculated (Table 1). For each alcohol there is a large increase in the amount of free phenolphthalein liberated. As regards phosphate liberation it appears that methyl, ethyl and *n*-propyl alcohols, at the concentrations used, cause no increase in phosphate liberation, whereas *iso*propyl alcohol produces a marked increase in the amount of phosphate liberated, and ethylene glycol a marked decrease.

DISCUSSION

Axelrod (1947) using an acid-optimum phosphatase prepared from orange juice, and monophenyl phosphate, *p*-nitrophenyl phosphate or sodium phenolphthalein phosphate as substrate, found that in the presence of certain alcohols, transference of phosphate from the substrate to the alcohol is catalyzed by a newly described enzyme. Such a reaction would account for the results described in the present communication, assuming that there are two enzymes present, an acid-optimum phosphatase and a transphosphorylase which catalyze the following reactions:

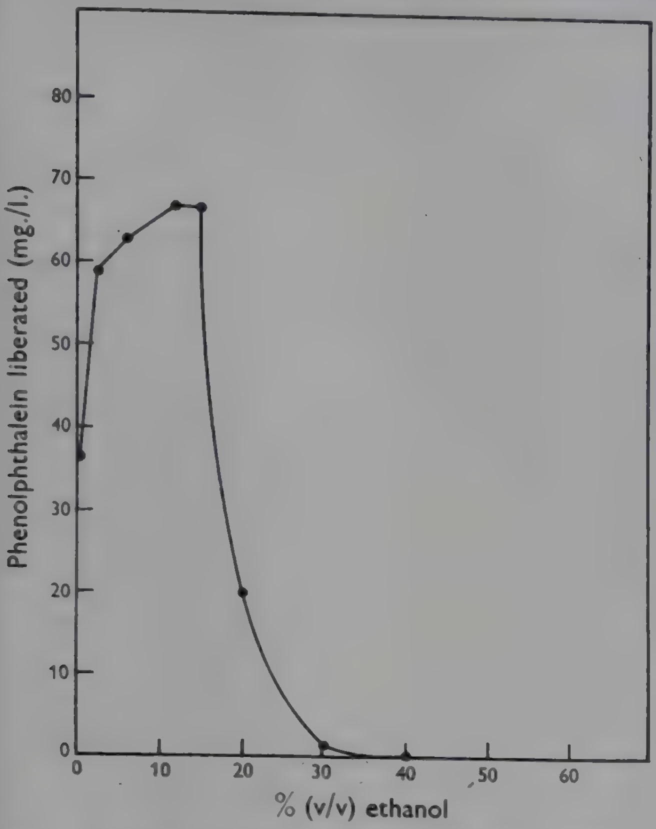

Fig. 1. The effect of different concentrations of ethanol on the production of free phenolphthalein by the action of prostatic phosphatase extract on pentasodium phenolphthalein diphosphate.

$$(a)\ \phi\text{-Phosphate} \xrightarrow{\text{Phosphatase}} \text{Phosphate} + \phi,$$

$$(b)\ \phi\text{-Phosphate} + A \xrightarrow{\text{Transphosphorylase}} A\text{-phosphate} + \phi.$$

ϕ = phenolphthalein; A = alcohol.

Thus, in the presence of an alcohol, there is an increase in the liberation of free phenolphthalein, but no such effect on phosphate liberation.

In the case of ethylene glycol, the transphosphorylase presumably competes more effectively than the phosphatase for the phenolphthalein phosphate, whereas the case of *iso*propyl alcohol suggests that the alcohol phosphate is split again. Fig. 1 indicates that the transphosphorylase is inactivated by 20% (v/v) ethanol.

SUMMARY

1. The formation of free phenolphthalein from pentasodium phenolphthalein diphosphate by prostatic phosphatase preparations acting at pH 5·4 and 37° is increased by methyl, ethyl, *n*-propyl and *iso*propyl alcohols, and ethylene glycol.

2. The formation of free phosphate under the above conditions is apparently unaffected by methyl, ethyl, and *n*-propyl alcohols, increased by *iso*propyl alcohol and decreased by ethylene glycol.

3. An interpretation of these results in terms of transphosphorylation is suggested.

I should like to thank Dr N. R. Lawrie for his help during this work, and Dr M. Dixon for many valuable discussions.

Table 1. *The effect of alcohols upon the amounts of phenolphthalein and phosphate liberated from pentasodium phenolphthalein diphosphate, by incubation with a prostatic extract*

(ϕ = phenolphthalein. A = alcohol. Inorganic phosphate is expressed as mg. P/l.)

Alcohol (%, v/v)	ϕ liberated (mg./l.)		P liberated (mg./l.)		Increase (%)	
	With A	Without A	With A	Without A	ϕ	P
15% ethyl	75·8	45·0	18·6	19·4	+ 66	− 4
	94·5	58·5	16·6	15·6	+ 62	+ 6
	91·0	52·5	20·4	18·5	+ 74	+10
10% methyl	89·0	52·0	16·3	17·1	+ 71	− 5
	97·0	54·0	15·7	16·9	+ 79	− 7
	90·0	49·2	15·4	15·9	+ 83	− 3
5% *n*-propyl	86·5	56·0	20·9	19·7	+ 53	+ 6
	85·0	56·0	21·6	22·4	+ 52	− 7
	90·0	58·0	20·3	18·9	+ 55	+ 6
10% *iso*propyl	88·5	56·5	27·7	20·7	+ 57	+34
	83·0	51·5	26·5	17·7	+ 61	+50
	88·5	54·5	25·4	18·2	+ 62	+39
20% ethylene glycol	92·5	53·5	18·0	20·0	+ 73	−10
	89·5	41·2	11·8	19·4	+117	−39
	94·5	51·5	15·7	22·2	+ 84	−30

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Reversibility of a Phosphoroclastic Reaction

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Utter, Lipmann & Werkman (1945) using C^{13} as a tracer showed reversibility of the phosphoroclastic reaction in *Escherichia coli* according to the equation:

$$CH_3COC^{13}OOH + H_3PO_4 \rightleftharpoons CH_3COOPO_3H_2 + HC^{13}OOH. \quad (1)$$

Esch. coli possesses an enzyme, hydrogenlyase, which reversibly performs the reaction:

$$HCOOH \rightleftharpoons H_2 + CO_2. \quad (2)$$

Reaction 1 occurring with reaction 2 involves carbon dioxide fixation.

Koepsell & Johnson (1942) obtained a cell-free extract from *Clostridium butylicum* which converts pyruvic acid into acetic acid, carbon dioxide and hydrogen, and requires phosphate for the reaction. Later Koepsell, Johnson & Meek (1944) found that inorganic phosphate is taken up and labile phosphate appears. The overall reaction may be illustrated:

$$CH_3COCOOH + H_3PO_4 \rightarrow CH_3COOPO_3H_2 + CO_2 + H_2. \quad (3)$$

The question arises whether formic acid occurs as an intermediate (Osburn, Brown & Werkman (1937) and Stiles, Peterson & Fred (1929)). This communication deals with the reversibility of reaction 3, and evidence is presented that formic acid is not an intermediate. Reaction 3 appears to differ fundamentally from the phosphoroclastic reaction of *Esch. coli*.

METHODS

Cl. butylicum (Beijerinck) Donker was used in this investigation. The organism was grown in a medium of 1 % glucose, 0·4 % yeast extract, 0·4 % peptone, 0·1 % beef extract, 0·05 % reduced iron, 10 % tap water and 1·25 % K_2HPO_4 and adjusted to pH 7 with HCl. The glucose and K_2HPO_4 were sterilized separately and added aseptically to the medium. The medium was inoculated with 100 ml. of an 18 hr. culture and incubated at 37° for 18–24 hr. The medium was strained through cheese cloth to remove most of the iron. The cells were harvested with a Sharples centrifuge. The yield varied from 1·5 to 2·5 g./l. of medium. A cell-free extract was prepared from these cells by the method of Utter, Kalnitsky & Werkman (1946). Experiments with this extract were conducted in 125 ml. manometric flasks with total volumes indicated in the tables.

Reversibility was determined by incubating pyruvic acid with a properly buffered enzyme preparation in the presence of one or the other of the products containing an excess of C^{13}. The reaction was stopped short of completion and the residual pyruvic acid was recovered, degraded and analyzed for excess C^{13}.

Separation of the pyruvic acid from the acetic acid was accomplished by two methods: (1) steam distillation as carried out by Utter *et al.* (1945), and (2) ether extraction of the acetic acid in the presence of $NaHSO_3$ which forms an ether-insoluble complex with pyruvic acid. The bisulphite complex was decomposed by boiling and the pyruvic acid removed by ether extraction.

When only the carboxyl carbon of pyruvic acid was desired for isotopic analysis, it was obtained by oxidation with ceric sulphate, which oxidizes pyruvic acid to acetic acid and CO_2. The acetic acid was separated by steam distillation and oxidized to CO_2 with $K_2S_2O_8$. For the isotopic analysis of each carbon atom in the pyruvic acid the latter was degraded first by yeast Lebedev juice which decarboxylates pyruvic acid to acetaldehyde and CO_2. The acetaldehyde was treated with NaOI, when the methyl group formed iodoform and the aldehyde group formic acid. The iodoform was oxidized by dry combustion to CO_2 and the formic acid was oxidized to CO_2 by the method of Osburn & Werkman (1932).

$CH_3C^{13}OOH$ was prepared by means of the Grignard reaction. $HC^{13}OOH$ was prepared by the method of Utter *et al.* (1945). $C^{13}O_2$ was added as $NaHC^{13}O_3$.

RESULTS

Fixation of $C^{13}O_2$ *in the carboxyl group of pyruvic acid.* When the buffered enzyme preparation was incubated with sodium pyruvate and $NaHC^{13}O_3$ under the conditions of Table 1, the residual pyruvate contained C^{13} only in the carboxyl group. The

Table 1. *Fixation of* $C^{13}O_2$ *in pyruvic acid by enzyme extract from* Clostridium butylicum

(Reaction mixture: 375 mg. lyophilized *Cl. butylicum* extract, 1·6 mmol. $NaHC^{13}O_3$ containing 5·43 % (atom) excess C^{13}, 2·0 mmol. pyruvate, 1·08 mmol. phosphate, pH 6·8; total volume, 30 ml. Time, 7 hr.; temp. 30°. Atmosphere, 100 % N_2.)

Pyruvic acid utilized (mmol.)	Excess of C^{13} in (atom %)		
	Residual CO_2	CH_3CO— of pyruvic acid	—COOH of pyruvic acid
0·66	1·96	0·00	1·98

C^{13} contents of the carboxyl carbon and the residual CO_2 indicate that an equilibrium was established. There was no excess C^{13} in the CH_3CO— group.

Effect of hydrogen on fixation of $C^{13}O_2$. Lipmann & Tuttle (1945) observed that molecular hydrogen inhibited the breakdown of pyruvic acid by the enzyme extract of *Cl. butylicum*. It was of interest to determine the effect of hydrogen on the fixation of $C^{13}O_2$ under conditions similar to those of the previous experiment except that the atmosphere consisted of 50% hydrogen and 50% nitrogen (Table 2). Fixation was substantially greater in the presence of hydrogen, although the amount of pyruvate dissimilated was less than in the presence of 100% nitrogen. Apparently the point of equilibrium in the presence of hydrogen is farther to the left, resulting in greater fixation.

Table 2. *Effect of hydrogen on fixation of* $C^{13}O_2$ *by* Clostridium butylicum

(Reaction mixture: 15 ml. enzyme extract, 1·6 mmol. $NaHC^{13}O_3$ (5·43% excess C^{13}), 2·0 mmol. pyruvate, 1·08 mmol. phosphate, pH 6·8; total volume, 30 ml. Time, 3 hr.; temp. 30°. Degradation by ceric sulphate.)

		Excess of C^{13} in (atom %)		
Atmosphere	Pyruvic acid utilized (mmol.)	Residual CO_2	CH_3CO— of pyruvic acid	—COOH of pyruvic acid
100% N_2	1·19	1·90	0·00	1·59
50% N_2 + 50% H_2	0·78	1·80	0·00	1·75

Exchange between $CH_3C^{13}OOH$ *and* $CH_3COCOOH$. If equation (3) is reversible, then before acetic acid could participate in it, it would first need to be phosphorylated to acetyl phosphate. Results of Table 3 show the results of adding $CH_3C^{13}OOH$ without an added energy-rich source of phosphate. In order to ensure adequate separation of the pyruvic acid from the $CH_3C^{13}OOH$, both the bisulphite-ether extraction method and the double steam distillation were employed on each sample. The pyruvic acid was degraded by the yeast carboxylase and by the ceric sulphate methods, and essentially the same C^{13} content was obtained by both methods. Results in Table 3 were obtained by the ceric sulphate method. The C^{13} content of the —CO— group was calculated from the content of the CH_3CO— group. The average values obtained with repeated experiments showed the same magnitude of fixation. The calculated value of C^{13} for the —CO— group is nearly in equilibrium with the residual $CH_3C^{13}OOH$. Results of an experiment carried out with whole cells (when possibly greater exchange might occur) did not significantly differ from those obtained with the enzyme extract.

Table 3. *Exchange between* $CH_3C^{13}OOH$ *and* $CH_3COCOOH$

(Reaction mixture: 6 ml. enzyme extract from *Cl. butylicum*, 1·1 mmol. $CH_3C^{13}OONa$ (4·65% excess C^{13}), 2·0 mmol. pyruvate, 1·08 mmol. phosphate buffer, pH 6·8; total volume, 30 ml. Time, 60 min.; temp. 30°. Atmosphere, 95% H_2 + 5% CO_2. Pyruvate dissimilated, 1·39 mmol.)

Reaction product examined	Excess C^{13} (atom %)
Residual CH_3COOH	0·11
Pyruvate:	
—COOH	0·00
CH_3CO—	0·04
—CO—	0·08 (calc.)

Effect of adenosine triphosphate (ATP) and acetyl phosphate on the exchange of $CH_3C^{13}OOH$. Utter *et al.* (1945) working with cell-free extracts of *Esch. coli* found that significant fixation of $CH_3C^{13}OOH$ in pyruvate occurred only in the presence of adenosine triphosphate. The high-energy phosphate was required apparently for the phosphorylation of the acetic acid. Table 4, Exp. 1, records the results of a typical experiment carried out in the presence of *ATP* and $CH_3C^{13}OOH$. Exp. 2 contained acetyl phosphate and adenylic acid in addition to the $CH_3C^{13}OOH$. In the presence of acetyl phosphate, adenylic acid and $CH_3C^{13}OOH$ (Exp. 2) the C^{13} content of the CH_3CO— group of the pyruvate was significantly higher than when $CH_3C^{13}OOH$ was used alone. The C^{13} content is also greater in the presence of *ATP* (Exp. 1); however, it is not as high

Table 4. *Effect of ATP and acetyl phosphate on* $CH_3C^{13}OOH$ *fixation*

(Each experimental mixture contained 5 ml. *Cl. butylicum* extract, 2 mmol. pyruvate, 1·06 mmol. $CH_3C^{13}OONa$ (4·65% excess C^{13}), 0·1 mmol. $MgCl_2$, and 1·08 mmol. phosphate buffer, pH 6·8. In addition, Exp. 1 contained 34 mg. adenylic acid and 75 mg. acetyl phosphate. Exp. 2 contained 195 mg. *ATP*. Atmosphere, 95% H_2 and 5% CO_2. Time, 1·5 hr.; temp. 30°.)

	Exp. 1, with adenylic acid + acetyl phosphate		Exp. 2, with adenosine triphosphate	
Grouping of pyruvate molecule which was estimated	Quantity (mmol.)	Excess C^{13} (atom %)	Quantity (mmol.)	Excess C^{13} (atom %)
—COOH	0·64	0·00	0·36	0·00
CH_3CO—	1·18	0·12	2·20	0·06
—CO—	—	0·24 (calc.)	—	0·12 (calc.)

as in Exp. 2. Assuming that the CH_3 group of the pyruvate contains no C^{13}, as indicated by degradation by yeast carboxylase and the iodoform reaction, calculation of the C^{13} content of the —CO— group gives values which are significant. The presence of the C^{13} in the —CO— group is expected from the fixation of $CH_3C^{13}OOH$.

Exchange between $HC^{13}OOH$ *and* $CH_3COCOOH$. Since it was questionable whether formic acid was an intermediate in the reaction, an experiment was carried out with pyruvic acid and $HC^{13}OOH$. If the reaction is similar to that in *Esch. coli*, exchange should occur with pyruvic acid. Since the occurrence of formic acid in the dissimilation of pyruvic acid by *Cl. butylicum* was in question, $HC^{13}OOH$ was used in an experiment with pyruvic acid. If the reaction with *Cl. butylicum* is similar to that of *Esch. coli*, C^{13} should be found in the residual pyruvic acid. The results (Table 5) show conclusively that formate does not exchange with pyruvate, and from the C^{13} content of the residual $NaHCO_3$ it is evident that the formate does not break down to H_2 and CO_2.

Table 5. *Absence of exchange between* $HC^{13}OOH$ *and* $CH_3COCOOH$

(Reaction mixture: 8 ml. *Cl. butylicum* extract, 2·0 mmol. phosphate buffer, pH 6·8; 1 mmol. $HC^{13}OOH$ (2·31% excess C^{13}), 2·0 mmol. pyruvate, 25 mg. *ATP*; total vol., 37 ml. Time, 60 min.; temp. 30°. Atmosphere, 50% H_2 and 50% N_2. Pyruvate dissimilated, 0·88 mmol.)

Reaction product examined	Excess C^{13} (atom %)
Residual $NaHCO_3$	0·00
Pyruvate:	
—COOH	0·00
CH_3CO—	0·00
Formate	2·32

DISCUSSION

The phosphoroclastic reaction in *Cl. butylicum* has been shown to be reversible. Formic acid was not found to be an intermediate. This appears to be the first demonstration of C_2 and C_1 addition where carbon dioxide, *per se*, is the C_1 compound fixed. Such a fixation clarifies the data of Brown, Wood & Werkman (1944) who found an excess of C^{13} in the carboxyl group of lactic acid formed by *Cl. butylicum* from glucose in the presence of $C^{13}O_2$. Since no appreciable amounts of C_4 compounds were detected, no significant fixation of the C_3+C_1 type could have occurred.

Rigorous proof that acetyl phosphate is an intermediate in the reaction remains to be demonstrated; however, the presence of an energy-rich phosphate source together with $CH_3C^{13}OOH$ does enhance the reversibility of the reaction. In the phosphoroclastic reaction of *Esch. coli*, reduction of carbon dioxide to formic acid occurs prior to the C_2 and C_1 addition. With *Cl. butylicum* there is either a simultaneous reduction with fixation, or fixation occurs with subsequent reduction. It is difficult to visualize the latter possibility with acetic acid or acetyl phosphate as the C_2 compound concerned in the fixation. The possibility remains that some C_1 reduced compound other than formic acid is a component of the system.

The observation that an atmosphere of hydrogen enhanced fixation of carbon dioxide in pyruvic acid strengthens the evidence for reversibility of the reaction.

SUMMARY

1. The conversion of pyruvic acid into acetic acid, carbon dioxide and hydrogen by *Clostridium butylicum* which requires phosphate has been demonstrated by the use of $NaHC^{13}O_3$ to be reversible.

2. It is shown that molecular hydrogen enhances fixation of carbon dioxide in this reaction.

3. Formic acid is not an intermediate and it is suggested that acetyl phosphate might be a component of the system, although no rigorous proof for this hypothesis has as yet been obtained.

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The Infrared Examination of Compounds Related to Ascorbic Acid

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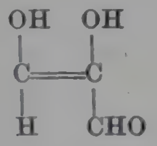
It is now realized that infrared absorption spectra can frequently be used with marked success in the differentiation of organic substances for the analysis of mixtures and for structural diagnosis. The present paper indicates briefly how it may be applied in one problem of interest in biochemistry.

Recent studies on vitamin C in plant materials have shown that its occurrence and/or functions may be better understood by reference to other closely related dienols, such as reductone (1), reductic acid (2), hydroxymaleic acid (3) and hydroxytetronic acid (4). These dienols, although possessing no antiscorbutic activity, all react with the indophenol dye normally used to estimate ascorbic acid (5). The first three, which are known to occur naturally, can be differentiated from ascorbic acid by various modifications of Lugg's (1942) formaldehyde method (cf. Mapson, 1943 *a*, *b*; Wokes, Organ & Jacoby, 1943). Hydroxytetronic acid cannot be so differentiated, neither can *iso*ascorbic acid (6), which is known to occur in certain commercial fruit juices. Moreover, the formaldehyde method does not differentiate between 1, 2, 3 and 4.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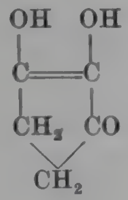
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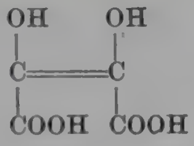
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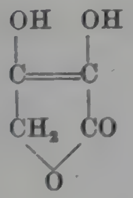
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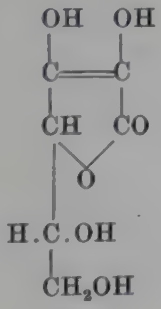
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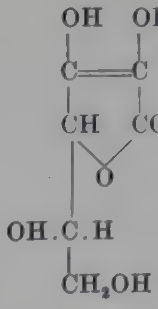
(6)

The infrared absorption spectra of these substances have therefore been measured in the hope of finding characteristic differences which may be used for analysis. The results given below show that such distinguishing features occur, and these may not only enable the analyses to be carried out, but may also provide a method which may help in following the course of plant processes.

EXPERIMENTAL

The spectra were measured between 2–14 μ. with an automatically recording single beam spectrometer, with rock salt prism (Whiffen & Thompson, 1945). A few measurements were also made in the region 5–7 μ. with a double beam spectrometer having a prism of calcium fluoride.

The compounds were obtained from Roche Products Ltd., except for dihydroxymaleic acid, which was a laboratory specimen. No impurities could be detected by their ultraviolet absorption spectra. A specially purified sample of reductic acid supplied by Prof. Reichstein was also used. The spectra of the solid powders were measured by grinding to a paste with a little paraffin in the standard manner. A satisfactory spectrum can be obtained with a few mg. of material. The paste was pressed between two small rock salt plates. Measurement of the spectrum of the solids had the advantage over solutions that the whole spectrum could be surveyed without serious disturbance by absorption bands of the solvent. For analytical work, it would be necessary to work with dilute solutions in some solvent chosen so as to have clear transmission in the region of the key absorption bands concerned. Over small regions of the spectrum shown in Fig. 1, paraffin has its own absorption bands, which may obscure those of the substance, but these are regions where the vibrations due to C—H bonds occur and it is certain that, from an analytical standpoint, they are unimportant. These spectral regions are marked *N* in Fig. 1.

RESULTS AND DISCUSSION

Fig. 1 shows the absorption spectra of the six compounds. The positions of the bands are shown on a wave-length scale (μ.), and also on a frequency scale, the customary units of reciprocal wave lengths, or waves/cm. (cm.$^{-1}$) being used. It is seen that, whilst in several cases there are absorption bands at the same positions, each substance has a different spectrum as a whole. Each compound has a band near 1650 cm.$^{-1}$, presumably due to the stretching vibration of the C═C bond. Some of the bands in the region 1100–1250 cm.$^{-1}$ may be connected with bending vibrations of the hydroxyl groups. The three compounds having a lactone ring show a band at about 1750 cm.$^{-1}$, which has previously been found common to many lactones (Richards & Thompson, 1947). In the region 2400–3600 there is interesting and varied absorption due to the stretching vibration of bonded-hydroxyl groups. It is noteworthy that the different compounds show marked differences in this respect, as has also been found in measurements on a series of sugars and their derivatives (Trotter & Thompson, 1947).

The spectra are unfortunately so complex that the analysis of a mixture of compounds 1, 2, 3, 4 and 5, if all were present together, would be very difficult.

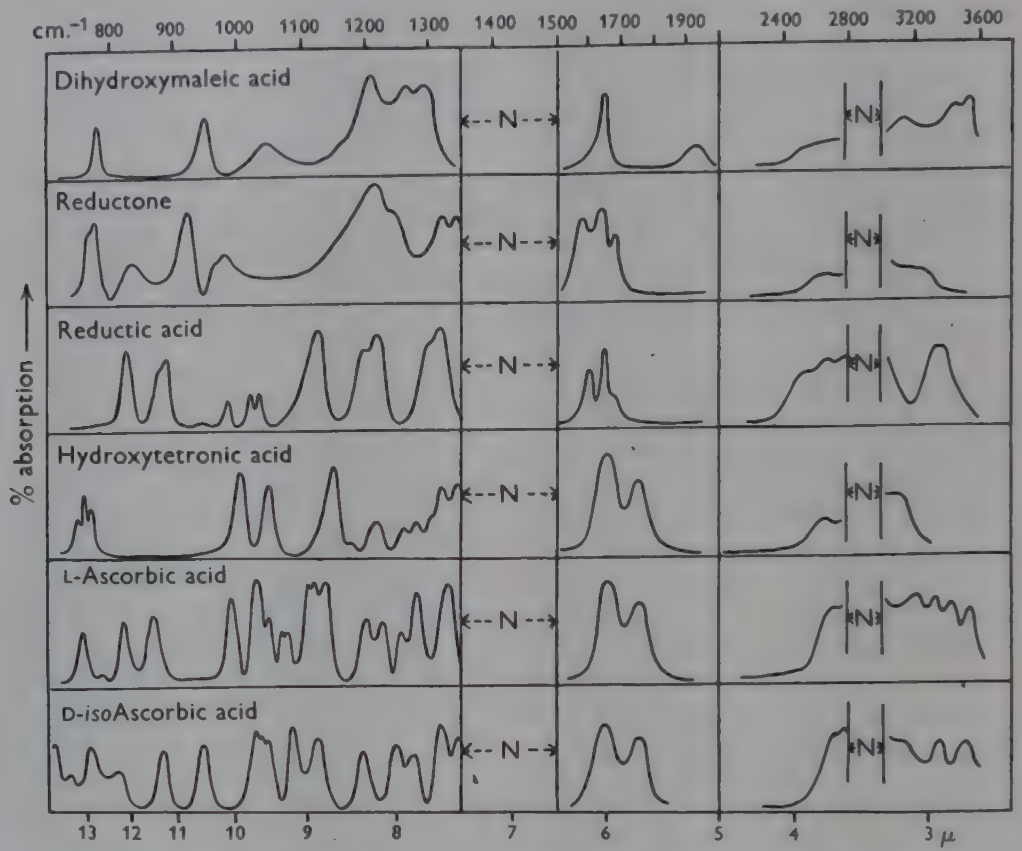

Fig. 1. Infrared absorption curves of compounds related to ascorbic acid. Region marked *N* obscured by bands of paraffin. Percentage absorption covers the range 100 %.

However, if only two or three components were present, it should be possible. For example, reductone could be determined in ascorbic acid by its band at 923 cm.$^{-1}$, or at 1580 cm.$^{-1}$, or ascorbic acid in reductone by its bands at 1030, 1120 or 1755 cm.$^{-1}$. Reductone could be differentiated from reductic acid by its bands at 777 or 923 cm.$^{-1}$, and reductic acid in reductone by its band at 1126 cm.$^{-1}$. A key band for dihydroxymaleic acid may be that at 950 cm.$^{-1}$. Differentiation of hydroxytetronic acid from reductic acid could be carried out with the bands between 750–900 cm.$^{-1}$. For the differentiation of ascorbic acid and *iso*ascorbic acid the bands at 991 and 941 cm.$^{-1}$ respectively seem very suitable.

As with all other analyses in the infrared, the sensitivity and accuracy will depend upon the precise mixtures to be analyzed, and will probably vary from case to case. We propose to use the method in studying the growth processes of certain plants, but it seems desirable to draw attention now to the possibilities of this type of measurement.

SUMMARY

The infrared absorption spectra of some compounds related to ascorbic acid have been measured between 2–14 μ. There are differences between the spectra which should make analyses of mixtures possible and more satisfactory than by existing chemical methods.

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The *in vitro* Synthesis of Glycogen in the Diaphragms of Normal and Alloxan-diabetic Rats

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Gemmill (1940, 1941) and Gemmill & Hamann (1941), confirmed by Hechter, Levine & Soskin (1941) and by Stadie (1944), showed that surviving rat diaphragm can synthesize glycogen from added glucose. The rate of this synthesis may be increased by addition of insulin *in vitro*. Following these findings the *in vitro* synthesis of glycogen from glucose as well as glycogenolysis by muscle were further investigated. It was proposed to examine (*a*) the influence of the composition of the medium, and especially the effect of potassium, on these reactions, (*b*) the influence of adrenaline, (*c*) the effect of insulin in normal animals, and (*d*) the synthesis of glycogen and glycogenolysis in diaphragms from alloxan-diabetic rats.

Cross & Holmes (1937) and Ostern, Herbert & Holmes (1939) found that liver slices can synthesize glycogen *in vitro* from added glucose. Hastings & Buchanan (1942) demonstrated that in rat-liver slices this synthesis takes place only in a potassium-rich, sodium-free medium, corresponding in ionic composition to intracellular fluid. In a medium resembling extracellular fluid no synthesis of glycogen takes place. Saxton & Miller (1944) showed that even under optimal conditions this synthesis of glycogen depends on the time interval which elapses between the death of the animal and the beginning of the experiment *in vitro*. On the basis of these experiments the synthesis of glycogen and glycogenolysis in rat-liver slices were studied *in vitro*, and were compared with the same processes in the diaphragm.

EXPERIMENTAL

Methods

Preparation of the diaphragm. Male albino rats of laboratory stock weighing 80–120 g. were used. The animals were anaesthetized with nembutal (pentobarbital) and bled. The diaphragm was quickly excised and two equal portions were weighed. In control experiments with both halves incubated under identical conditions, we observed the same deviations as were reported by Gemmill (1940). Dividing the diaphragm of older rats into three or more equal parts proved to be unreliable.

Media for incubation. (*a*) *Buffered salt solutions.* The diaphragm samples were incubated according to Gemmill (1940) at 38° in a medium described by Gey & Gey (1936) and modified by Gemmill (1940). Its composition was: 0·7 % NaCl, 0·037 % KCl, 0·227 % $NaHCO_3$, 0·017 % $CaCl_2$, 0·015 % Na_2HPO_4, and 0·003 % KH_2PO_4 (all % w/v). This medium was similar to the solution described by Van Dyke & Hastings (1928), and resembled body fluid in its ionic composition. It is referred to here as 'extracellular solution'. In our experiments modifications of this solution by addition or removal of an ion were compensated for by corresponding changes in NaCl content. With a modification of this solution, containing only 0·74 % NaCl, 0·015 % Na_2HPO_4 and 0·003 % NaH_2PO_4, we obtained optimal synthesis of glycogen by rat diaphragm *in vitro*. This solution is called 'phosphate buffered saline'. The glucose content of the media was usually 100 mg./100 ml., the concentration most favourable according to Hechter *et al.* (1941) for the demonstration of the insulin effect.

(*b*) *Serum.* For 'normal serum' pooled human serum was generally used, its glucose content being adjusted to 100 mg./100 ml. No differences were found with respect to synthesis of glycogen between sera of various animals, and fresh rabbit or rat sera were also used. When comparing synthesis in normal and diabetic serum, only freshly prepared rat serum was used, and the glucose content of the normal serum was adjusted to that of the diabetic serum. In another series of experiments the synthesis of glycogen in normal serum was investigated at a glucose concentration of 800 mg./100 ml., this being the mean serum glucose content in a series of alloxan-diabetic rats. The diaphragms of normal and alloxan-diabetic rats are termed 'normal diaphragm' and 'diabetic diaphragm', respectively, and the sera of these animals are similarly designated.

Bicarbonate-containing buffer media and sera were aerated with a gas mixture containing 95 % O_2 and 5 % CO_2, the bicarbonate-free phosphate-buffered saline with O_2. All solutions proved to be sufficiently buffered, and the experiments were carried out at a pH of approximately 7·4. No significant changes in pH were found after 3 hr. incubation with diaphragm even in unbuffered 0·9 % NaCl. A concentrate of rat serum of approximately double strength was prepared according to Kanner (1946) by means of purified gelatin.

Measurement of glycogen synthesis. As measure of glycogen synthesis the difference in glycogen content between one test sample incubated for 6 min. and the other for 3 hr. is used. This value is termed 'glycogen synthesis value'. The influence of various factors, such as ionic composition and additions of drugs, was measured in most of our experiments by the difference in the glycogen content of two samples after 3 hr. incubation, one sample being incubated in the usual medium and the other in medium modified in the appropriate manner. This value is called 'glycogen difference value'. The values are expressed in mg. increase/ 100 mg. diaphragm. The 'difference value' is also expressed as the percentage increase or decrease in glycogen.

The incubation of liver slices. Experiments on liver slices were carried out in the 'intracellular solution' described by Hastings & Buchanan (1942). The incubation time in these experiments was 4 hr., and glucose concentrations used were 0·25, 0·5 and 1%.

Experiments on glycogenolysis. These experiments were carried out in the extra- and intracellular solutions mentioned above without the addition of glucose. Serum was freed of sugar by incubation with yeast for 30 min. Glycogenolysis is expressed as the decrease in the glycogen content after 3 hr. incubation. Since the rate of glycogenolysis is dependent on the initial glycogen content, the values are given as the percentage decrease in glycogen as well as in mg./100 mg. tissue.

Respiration measurements. Determinations of oxygen uptake and respiratory quotient were carried out by Warburg's direct method (Dixon, 1943) in phosphate-Ringer. Measurements were made over periods varying from 2 to 3 hr. and the average value for 1 hr. was recorded.

Chemical procedures. Glycogen determinations in the diaphragm were carried out by a micromodification of Pflüger's method, employing liver slices according to Good, Kramer & Somogyi (1933). Glucose was estimated according to Shaffer, Hartmann & Somogyi (Somogyi, 1937).

Production of diabetes. Diabetes was induced by intravenous injection of 6–8 mg. of alloxan per 100 g. rat. Animals in various stages of diabetes were used, varying from those with severe coma to those with latent diabetes which exhibited glycosuria only on carbohydrate-rich food. Ordinary insulin (Squibb) was used, 2 units usually being added to 3 ml. of medium; this quantity corresponds approximately to the mean dose used by Gemmill. The ergotamine tartrate employed was Gynergen (Sandoz).

RESULTS

(1) *Influence of the composition of the medium, and especially of the potassium content on glycogen synthesis and glycogenolysis*

Removal of potassium from the 'extracellular solution' resulted in an increased synthesis of glycogen from glucose in rat diaphragm. Not only was the presence of calcium unnecessary for the synthesis of glycogen, but in a calcium-free medium the synthesis was even slightly improved, the increase being 20% ± 8 in 13 experiments. NH_4^+ had the same inhibiting effect as potassium. Optimal synthesis was observed in our experiments in phosphate-buffered saline. Two- or threefold increase in the potassium content of the 'extracellular solution' nearly always resulted in complete inhibition of glycogen synthesis (Table 1). In the 'intracellular' potassium-rich, sodium-free medium, described by Hastings & Buchanan (1942), intensive glycogenolysis occurred instead of synthesis, and was already manifest after 15 min. On the other hand, as demonstrated by these authors, glycogen synthesis in rat-liver slices takes place only in the intracellular medium. If in these experiments with liver slices part of the KCl is replaced by NaCl, glycogen synthesis is inhibited, but still demonstrable. With 'intracellular solution' glycogen can still be synthesized by liver *brei*, but only at the high glucose concentrations of 1·5–2%. In 13 experiments a mean increase of 0·63 mg. ± 0·19/g. liver *brei* was observed. Under no conditions could synthesis of glycogen be obtained in diaphragm *brei*. Contrary to our findings in the diaphragm, Ostern *et al.* (1939), working with liver slices, noted that the removal of calcium resulted in inhibition of glycogen synthesis.

Glycogen synthesis in the diaphragm was optimal at pH 7·4, and decreased by 13% ± 7 at pH 6·8. If serum was used as medium the synthesis of glycogen in the diaphragm was of the same order of magnitude as in phosphate-buffered saline (Table 1). Although the potassium content of serum is the same as that of the 'extracellular solution', no inhibitory effect could be observed with serum. Two- or threefold increase of the serum potassium, however, did result in a decreased synthesis of glycogen. Replacing one third to one fifth of the 'extracellular solution' by serum improved the synthesis of glycogen. No increase in synthesis could be induced by addition of serum to phosphate-buffered saline.

Glycogenolysis was of similar magnitude in phosphate-buffered saline and in serum. Much more intense glycogenolysis was observed with the 'extracellular solution' (Table 2).

Table 1. *Synthesis of glycogen in normal rat diaphragm in various media compared with synthesis in the 'extracellular solution'*

		Glycogen difference	
No. of exp.	'Extracellular solution', compared with	(mg./100 mg. tissue)	Increase or decrease (%)
9	'Extracellular', potassium-free	+0·065 ± 0·013	+48 ± 13
6	'Extracellular' + 0·01–0·015 M-KCl	−0·046 ± 0·020	−15 ± 6
10	Serum	+0·057 ± 0·018	+56 ± 25
6	'Extracellular' + 0·2–0·33 vol. of serum	+0·089 ± 0·018	+28 ± 6
12	Serum + 0·01–0·015 M-KCl	−0·046 ± 0·018	−13 ± 6
13	Phosphate saline	+0·120 ± 0·027	+87 ± 25

Table 2. *The influence of different media on glycogenolysis in rat diaphragm*

Medium	No. of exp.	Decrease in glycogen (mg./100 mg. tissue)	Decrease in glycogen (% decrease)
'Extracellular'	10	−0·126±0·028	−48±2
Phosphate saline	7	−0·049±0·017	−17±4·5
Serum	11	−0·068±0·024	−25±4

(2) *The influence of adrenaline*

The addition of adrenaline hydrochloride at a concentration of 0·33 μg./ml. regularly decreased glycogen synthesis. At concentrations of 0·033 μg./ml. the effect was irregular and at 0·0033 μg./ml. it disappeared. Inhibition could already be observed after 1 hr. incubation. Ephedrine at a concentration of 3·3 μg./ml. had the same effect as adrenaline, while tyramine at the same concentration did not change the rate of glycogen synthesis. The effect of adrenaline was not dependent on the presence of potassium. Ergotamine tartrate (8·3–10 μg./ml.) abolished the inhibitory effect of adrenaline on glycogen synthesis. Since ergotamine alone inhibits the synthesis of glycogen it must be assumed that its action under the conditions of this experiment is due to its antagonizing effect on adrenaline. Insulin weakened the effect of adrenaline in 8 out of 13 experiments (Table 3).

Table 3. *The influence of adrenaline and the antagonizing effect of ergotamine tartrate on synthesis of glycogen in diaphragm*

No. of exp.	Samples incubated	Glycogen difference (mg./100 mg. tissue)	Glycogen difference: Increase or decrease (%)
8	With and without adrenaline	−0·085±0·027	−44±7
8	With adrenaline, with and without ergotamine	+0·060±0·012	+31±5·5
3	With and without ergotamine	−0·060±0·014	+16±4

The effect of adrenaline on glycogenolysis is of the same order of magnitude as its effect on glycogen synthesis. Adrenaline at a concentration of 0·33 μg./ml. increased glycogenolysis by 47%±7 (6 experiments). Cross & Holmes (1937), working with liver slices, first demonstrated the inhibition *in vitro* of the synthesis of glycogen by adrenaline, albeit only at a concentration of 50 μg./ml. Bendall & Lehmann (1941) produced a twofold effect of adrenaline at a concentration of 0·45 μg./ml. on the synthesis of glycogen in liver slices: an initial reduction and a subsequent increase. No clear-cut effect of insulin on glycogenolysis produced by adrenaline could be observed.

(3) *The influence of insulin on glycogen synthesis and glycogenolysis*

The experiments of Gemmill (1940) on the influence of insulin on the synthesis of glycogen, in the solution used by him, were confirmed, and the same effect was also demonstrated in phosphate-buffered saline. In serum, however, no additional increase of synthesis could be induced by insulin (Table 5). No influence of insulin on glycogenolysis could be observed, either in the 'extracellular solution' or in phosphate-buffered saline.

(4) *Glycogen synthesis and glycogenolysis in the diaphragm of alloxan-diabetic rats: the influence of insulin*

In the 'extracellular solution' the synthesis of glycogen by diabetic diaphragm was certainly not less than that of normal diaphragm and may have been somewhat higher. In phosphate-buffered saline no difference was found between glycogen synthesis by normal and diabetic diaphragm. In serum the synthesis by the diabetic diaphragm was somewhat lower but not to a significant degree (Table 4). Glycogenolysis in the 'extracellular solution' was of the same order of magnitude in diabetic and normal diaphragms.

The addition of insulin to the 'extracellular solution' resulted in synthesis values of 0·130 mg./100 mg. diaphragm for the diabetic and 0·150 for the normal rat, synthesis values without insulin being 0·079 and 0·055, respectively. In phosphate-buffered saline the effect of insulin was markedly lower on the diabetic than on the normal diaphragm (Table 5).

Table 4. *Synthesis of glycogen in diaphragm of alloxan-diabetic and normal rats in different media*

Medium	Alloxan-diabetic rats: No. of exp.	Alloxan-diabetic rats: Synthesis (mg./100 mg. tissue)	Normal rats: No. of exp.	Normal rats: Synthesis (mg./100 mg. tissue)
'Extracellular'	23	0·079±0·015	18	0·055±0·013
Phosphate saline	13	0·080±0·017	14	0·109±0·020
Pooled normal serum	31	0·057±0·012	29	0·084±0·012

Table 5. *The influence of insulin on synthesis of glycogen in diaphragm of alloxan-diabetic and normal rats*

	Alloxan-diabetic rats		Normal rats	
Medium	No. of exp.	Glycogen difference (mg./100 mg. tissue)	No. of exp.	Glycogen difference (mg./100 mg. tissue)
Phosphate saline	18	+0·044±0·037	20	+0·144±0·030
Normal pooled serum	22	+0·059±0·015	20	−0·032±0·022

In a further series of experiments the synthesis values of normal and of diabetic diaphragms, incubated in diabetic and in normal serum (glucose concentration 0·8%), were determined. The synthesis of glycogen in diabetic diaphragm was significantly diminished in diabetic serum, whereas in normal serum normal synthesis values were obtained. The synthesis of glycogen in normal diaphragm was markedly depressed in diabetic serum.

Table 6. *Synthesis of glycogen in alloxan-diabetic and normal diaphragm incubated with alloxan-diabetic and normal serum and the effect of insulin*

Diaphragm	Serum	No. of exp.	Insulin	Synthesis (mg./100 mg. tissue)	No. of exp.	Difference (mg./100 mg. tissue)
Diabetic	Diabetic	18	−	0·122±0·022		
Normal	Normal	20	+	0·215±0·020		
Diabetic	Normal	12	−	0·204±0·036		
Normal	Diabetic	15	−	0·151±0·021†		
Diabetic	Diabetic	9	+	0·410±0·052	7	+0·171±0·040
Diabetic acidotic	Diabetic	6	+	0·135±0·027		
Diabetic	Diabetic, insulin-treated *in vivo*	7	−	0·290±0·050		
Diabetic	Diabetic (1/2)+normal serum concentrate (1/2)	7	−	0·201±0·017		
Normal	Normal	10	+	0·373±0·056	10	+0·076±0·046

† One experiment exhibited a distinctly higher synthesis value of 0·616 and is not included in this table.

Addition of insulin to diabetic serum produced a marked elevation of the synthesis value in diabetic diaphragm. Since the usual dose of 2 units of insulin was rather high, some experiments were carried out with one-tenth of this dose, which was found to be still effective. Furthermore, insulin led to an increase in synthesis by normal diaphragm in normal serum at a glucose concentration of 0·8%. The effect of insulin under these conditions was, however, irregular; only in 5 out of 10 experiments could an increase in synthesis be induced. Incubation in the serum of alloxan-diabetic insulin-treated rats, or in a medium containing equal volumes of diabetic and of normal serum concentrate, resulted in an increase in synthesis in the diabetic diaphragm. Addition of insulin in the usual or even higher dose had no effect on the synthesis in the diaphragm of alloxan-diabetic acidotic rats (Table 6).

(5) *Respiration of normal and diabetic diaphragms**

In phosphate-Ringer solution, with and without glucose, the respiration of diabetic diaphragm was markedly higher than that of normal diaphragm. This result requires an explanation. Since glycogen synthesis differed in serum from that in phosphate-buffered saline, respiration experiments were carried out in normal and diabetic serum. In normal serum the respiration of normal diaphragm was higher than that of diabetic diaphragm, whereas in diabetic serum no difference could be observed. In experiments with serum only the oxygen uptake was measured, since the direct Warburg method proved unsuitable for the estimation of the CO_2 output (Table 7).

(6) *Glycogen synthesis in liver slices of alloxan-diabetic rats*

In this experiment we were handicapped by the fact that the livers of diabetic animals after fasting still contained appreciable quantities of glycogen (Tuerkischer & Wertheimer, 1946), and a glycogen content of 0·2–0·3% is considered to be the upper

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* The respiration experiments were carried out by Mrs I. Salomonowicz-Parnes.

Table 7. *Respiration of diaphragm of alloxan-diabetic and normal rats*

	Alloxan-diabetic rats				Normal rats			
		O_2	CO_2			O_2	CO_2	
Medium	No. of exp.	(μl./mg. dry wt./hr.)		R.Q.	No. of exp.	(μl./mg. dry wt./hr.)		R.Q.
Phosphate-Ringer	10	−5·98±0·29	4·83±0·27	0·81±0·010	17	−3·68±0·80	2·68±0·61	0·73±0·016
Phosphate-Ringer +0·1% glucose	16	−6·71±0·33	5·90±0·25	0·88±0·013	16	−4·47±0·67	3·90±0·68	0·87±0·024
Normal serum, glucose average 0·12%	9	−5·41±0·22	—	—	9	−6·85±0·29	—	—
Diabetic serum, glucose average 0·5%	5	−5·42±0·32	—	—	9	−5·40±0·22	—	—

limit for the demonstration of synthesis. In acidotic alloxan-diabetic rats, where no glycogen is to be found in the liver, synthesis of glycogen from added glucose was markedly diminished. In these animals the liver is already severely damaged, as shown by the high fat content. Rats suffering from a mild diabetes with normal fasting blood sugar, on the other hand, did not differ from normal rats in regard to the synthesis of glycogen.

DISCUSSION

The effect of potassium on the synthesis of glycogen and glycogenolysis

Potassium in concentrations which are present in extracellular fluid inhibits the synthesis of glycogen from added glucose in rat diaphragm, and complete removal of potassium produces optimal conditions for synthesis. In the liver, on the other hand, synthesis of glycogen will take place only in a medium containing appreciable amounts of potassium (Hastings & Buchanan, 1942), and with this organ the 'extracellular' medium favours glycogenolysis. The same medium thus has an opposite effect on identical processes in liver and muscle. Since even physiological quantities of potassium affect synthesis and favour glycogenolysis in the diaphragm, one must assume that potassium is of physiological importance in the regulation of synthesis and breakdown of glycogen. It is known that part of the body potassium occurs in a free condition, and is able to move about in response to changes in membrane equilibrium. For instance a decrease in the potassium content of working muscle has been adequately demonstrated by Fenn (1940) and Hahn & Hevesy (1941). Fenn assumes that, on its way from muscle to liver, potassium migrates together with lactic acid, and on its way from liver to muscle with glucose. Boyer, Lardy & Phillips (1942, 1943) explain the effect of potassium as being due to an acceleration of the transfer of phosphate from 2-phosphopyruvate to the adenylic acid system. Substitution of potassium by the ammonium ion in equimolar concentration had the same effect. In the experiments of Boyer *et al.* calcium counteracted the effect of potassium. In our experiments, on the other hand, no antagonism between potassium and calcium was found. This might be because the above authors used minced or homogenized muscle, while in our experiments tissue was used which had suffered minimal damage and calcium might be unable to enter the intact cell. However, the role of potassium in the phosphorylating process of the adenylic acid system does not provide an explanation of the opposite effect of this ion on identical processes in liver and muscle tissue. Serum, on the other hand, contains a factor which counteracts the effect of potassium. Owing to the presence of this factor, serum is an optimal medium for glycogen synthesis in the diaphragm. The cause of this effect is still to be explained.

The effect of adrenaline on the synthesis and breakdown of glycogen

The complete inhibition of synthesis and the increase of glycogenolysis in rat diaphragm on addition of small doses of adrenaline is in accord with the findings of Hegnauer & Cori (1934), Cori & Cori (1936) with muscle and Nachmansohn (1937) with muscle *brei*. These authors found that adrenaline increased the formation of hexosemonophosphate and of lactic acid in frog muscle under aerobic as well as under anaerobic conditions. However, whereas in their experiments these metabolic changes were not inhibited by ergotamine, in our experiments the inhibition of the synthesis of glycogen by adrenaline was completely abolished by ergotamine; this may be considered as evidence that in this case adrenaline acts by way of the sympathetic nervous supply still present in the preparation.

Synthesis of glycogen in alloxan-diabetic rats

The *in vitro* synthesis of glycogen in diaphragm from alloxan-diabetic rats showed the following characteristics: the difference between normal and diabetic diaphragm became apparent only when both serum and diaphragm from alloxan-diabetic rats were used. Under such conditions there was a marked decrease in synthesis with diabetic animals, whilst addition of insulin to diabetic serum greatly increased the synthesis. A similar increase in synthesis could be produced by incubation with serum of insulin-treated alloxan-diabetic rats, with serum of normal rats, or by addition of a normal serum concentrate to the diabetic serum. Incubation with diabetic serum, on the other hand, diminished synthesis of glycogen in the normal diaphragm.

This effect of insulin on diabetic diaphragm incubated with diabetic serum failed to appear when the diaphragms of severely diabetic rats with acidosis were used. In these experiments the known resistance of comatose alloxan-diabetic rats to insulin (Kaplan, Franks & Friedgood, 1945) was corroborated *in vitro*. In buffered salt solutions, however, no significant difference existed between the synthesis of glycogen in normal and diabetic diaphragms. The effect of insulin on the synthesis in diabetic diaphragm with these media was even smaller than that with normal diaphragm. The insulin effect, always present in buffered salt solution, could not be produced in the normal diaphragm incubated with normal serum. In the diabetic diaphragm this effect was definitely present.

An explanation of most of these findings can be given as follows: in normal serum a factor favouring synthesis of muscle glycogen, possibly insulin, is present, and synthesis cannot be improved by addition of insulin. At high glucose concentrations the amounts of this factor present may not be sufficient, and under these conditions addition of insulin may produce an increase in the synthesis of glycogen. In buffered salt media, therefore, an insulin effect could always be produced. In diabetic serum the concentration of this factor is either diminished, or the factor is absent and synthesis of glycogen is therefore decreased. However, this synthesis can be increased by addition of insulin, or by incubation with serum of insulin-treated alloxan-diabetic rats, or by addition of a normal serum concentrate. We can offer no explanation for the diminished effect of insulin in the diabetic diaphragm in buffered salt media.

So far mention has been made only of a factor present in normal and absent from diabetic serum which favours the synthesis of glycogen. But there still exists the possibility that in normal serum two substances may be present, one favouring and the other inhibiting synthesis. Such an assumption would explain the fact that syntheses by the normal diaphragm in the optimal buffered saline and in serum are of the same order of magnitude. Further experiments are required to investigate the validity of this assumption. On the other hand, it is probable that alloxan diabetes may also produce changes in muscle, even if such changes are not apparent in buffered salt media. That a disturbance must exist in the diabetic diaphragm itself is proved by the absence of the insulin effect in the diaphragm of alloxan-diabetic comatose rats incubated with serum from alloxan-diabetic non-comatose rats, and by the effectiveness of insulin on the diabetic diaphragm incubated with normal pooled serum.

In any case these results support the view that important factors influencing the normal carbohydrate metabolism of tissue slices are present in serum. Experiments on synthesis of glycogen as well as on respiration demonstrate that the use of serum in place of buffered salt media may lead to entirely different results (Shipley, 1944). Disturbances in metabolism may become detectable *in vitro* only when the experiments are carried out in the abnormal pathological environment and not in indifferent salt solutions.

SUMMARY

1. Glycogen synthesis and glycogenolysis by rat diaphragm and liver have been studied *in vitro*.

2. Potassium, even in physiological concentrations, inhibited synthesis of glycogen from added glucose by rat diaphragm. At higher concentrations of potassium glycogenolysis took place instead of synthesis. For glycogen synthesis in rat-liver slices, however, it is essential to have potassium present in the medium.

3. Adrenaline completely inhibited synthesis of glycogen in diaphragm and favoured glycogenolysis, whilst ergotamine abolished the effect of adrenaline.

4. Insulin favoured synthesis of glycogen from added glucose in all buffered salt media. With serum, however, no additional synthesis of glycogen could be induced by addition of insulin.

5. Incubation with 'diabetic' serum decreased the synthesis of glycogen in the 'diabetic' as well as in the 'normal' diaphragm. The synthesis in the 'diabetic' diaphragm could be increased by addition of insulin or of a normal serum concentrate to 'diabetic' serum, or by incubation with serum of insulin-treated alloxan-diabetic rats. In normal pooled serum a distinct increase in synthesis was obtained in 'diabetic' but not in 'normal' diaphragm by addition of insulin.

6. No differences, however, between the synthesis of glycogen in diaphragms of alloxan-diabetic and normal rats were observed in the different salt media. The effect of insulin in the 'diabetic' diaphragm in buffered salt media was even smaller than that observed in 'normal' diaphragm.

7. Glycogenolysis in diaphragm was favoured by potassium but unaffected by insulin. The order of magnitude of glycogenolysis was the same in 'normal' and 'diabetic' diaphragm.

8. Metabolic changes of tissues *in vitro* may become manifest only in a correspondingly changed medium and may be undetectable in an indifferent salt medium.

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Hydrochloric Acid Production by Isolated Gastric Mucosa*

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WITH AN APPENDIX BY R. E. DAVIES AND F. J. W. ROUGHTON

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The experiments described in this paper were undertaken to throw light on the mechanism of hydrochloric acid production in the stomach. Isolated mucosa was used because it is easier to control the experimental conditions in excised tissue than in the intact organism.

The oxyntic cells of rats secrete continuously (Friedman, 1943), hence the starting materials for the production of HCl must come eventually from the blood and must be neutral. Several mechanisms which produce hydrogen ions have been envisaged and can be divided roughly into two types. (1) Those producing the acid together with approximately neutral compounds. (2) Those producing the acid together with basic compounds (Fig. 1). Many manometric experiments have been carried out in Warburg cups with sheets of sheep, toad and frog gastric mucosa to test the possibility that mechanisms of type 1 are involved. In no case was evidence obtained that HCl was being formed, and liberating CO_2 from the HCO_3^- of the medium, although the rate of respiration of the tissues was normal and was increased by histamine. Since several glands can function *in vitro* (Delrue, 1930; Deutsch & Raper, 1936) it seemed unlikely that the failure to detect acid was due to the inability of the mucosa to secrete it. These negative results suggest that mechanisms of type 1 are not operative, as would be expected if the old hypothesis of Bence Jones (1845) that 'the formation of acid sets free alkali' were true, assuming that the acid and alkali are formed in equal amount as in the type 2 mechanisms.

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* A part of the present work was communicated to the Biochemical Society (Davies, 1946).

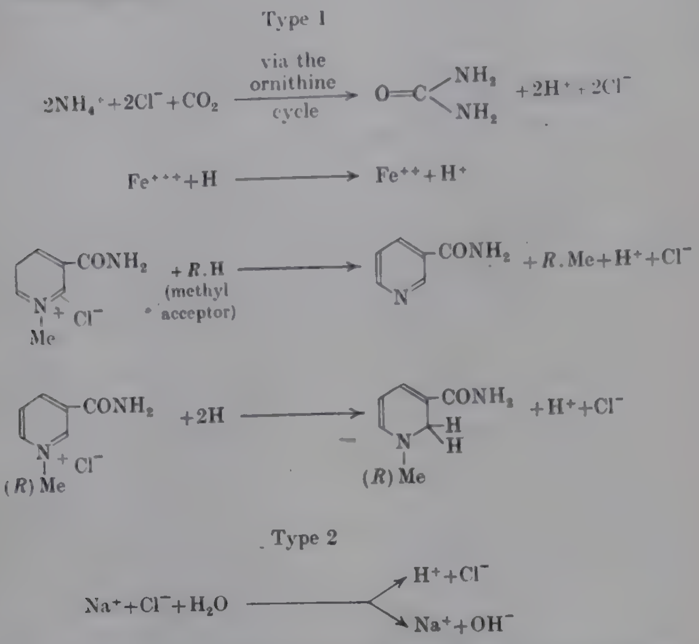

Fig. 1. Some biochemical reactions producing hydrogen ions. Type 1. Those producing the acid and approximately neutral compounds. Type 2. Those producing the acid and basic compounds. Type 1 mechanisms which are also reversed within the cell become type 2 mechanisms.

Experiments were devised to combine collection and estimation of the secretions with manometric measurements of the respiratory activity of the tissue; they show that when gastric mucosa secretes acid, an equivalent amount of alkali is also produced in the cell, which normally is at once neutralized by CO_2 and secreted into the surrounding medium.

METHODS

Saline media. The physiological salt solution (bicarbonate saline) of Krebs & Henseleit (1932), gassed with 5% (v/v) CO_2 + 95% (v/v) O_2, or 5% CO_2 + 95% N_2, was used during measurements of the balance of the O_2 uptake and the CO_2 output, or of the anaerobic glycolysis of sheep gastric mucosa, and the phosphate saline of Krebs (1933) during experiments in 100% O_2. The glucose concentration was 0·2 or 0·4%. In experiments with amphibian tissue these two solutions were used, after the addition of 0·29 vol. of water, and contained 0·2% (w/v) glucose. Another solution was used in the preparation of the mucosa and during some experiments in 100% O_2. It was a basal saline similar to amphibian bicarbonate saline, but contained 0·004 M-HCO_3^-.

Preparation of the gastric mucosa. Sheep, frog and toad gastric mucosae were used. The abomasum was removed from the sheep within 5 min. of electric stunning, and carried rapidly to the laboratory in ice-cold 0·16 M-NaCl solution. After thorough washing a mucosal flap was dissected away, opened out and freed from submucosa. Pieces of tissue with a dry weight of 20–40 mg. were cut off and used at once. They were about 0·3–0·6 mm. thick, and did not curl up tightly.

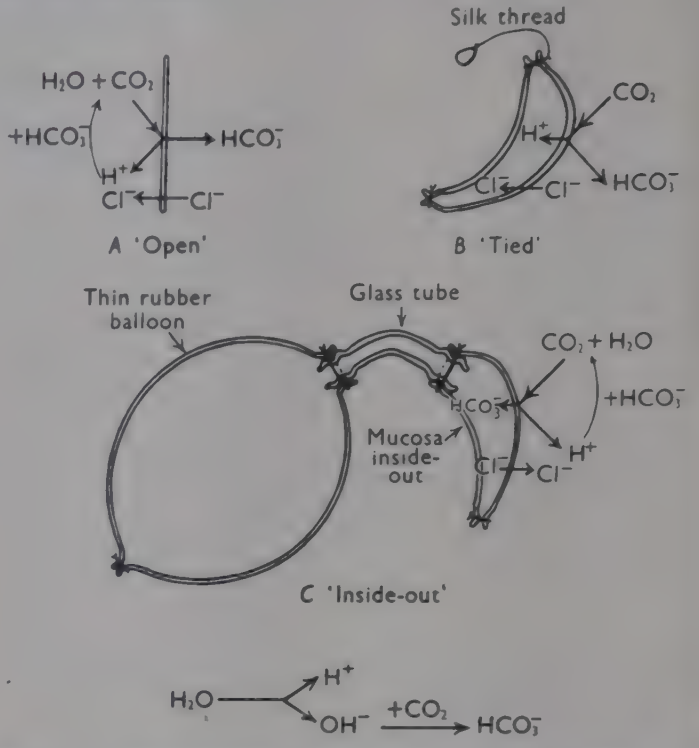

Fig. 2. Diagrammatic representation of the overall reactions in a type 2 mechanism in *A* 'open', *B* 'tied' and *C* 'inside-out' frog gastric mucosa in saline media containing bicarbonate ions. In *A* and *C* the CO_2 liberated from the medium by the H^+ is equivalent to that needed to neutralize the OH^-, so no supplies of CO_2 are needed. In *C* the decrease of the HCO_3^- content in the external fluid is equivalent to the increase in the internal fluid. In *B* the acid secretion is collected inside the mucosal bag and supplies of CO_2 are needed to neutralize the OH^-.

Frogs (*Rana temporaria temporaria* L.) and toads (*Bufo bufo* (L.)) were captured locally and housed in a concrete froggery at 15–17° after the web had been tattooed with Indian ink for identification. An excess of cockroaches or worms was provided for food. The amphibia were pithed as required, and the stomach dissected out after cutting the oesophagus and duodenum. It was emptied by gentle squeezing and washed by passing 5 ml. of basal saline through it from a pipette. In none of more than 400 cases has any residual acid been found in the stomach after this procedure. The following technique was evolved for isolating the intact gastric mucosa. The stomach, with the greater curvature upwards, was gently pressed with the left thumb and index finger to tighten the muscle coats. A small longitudinal incision was made with a razor, and continued to both ends with fine scissors without damaging the mucosa. Starting from the pyloric end, the intact tube of mucosa was separated from the muscles with fine forceps and pulled free. It was then blotted and rapidly weighed on a torsion balance; with care the weights were reproducible to 1 mg. During the dissection the tissue was frequently damped with basal saline.

Amphibian tissue was used in three different ways: 'open', 'tied' and 'inside-out'. For experiments with open tissue the mucosa was cut along the greater and lesser curvatures and used directly. Tied tissue was used when collection of the secretion was required; the pyloric end of the mucosa was closed with fine silk thread, and the lumen completely emptied by gentle stroking with curved forceps. The cardiac end was next tied, and a loop made in the silk with which to hang the preparation on the hook of the torsion balance. Excess silk was cut off and the tissue blotted and reweighed. After clipping off the spare tissue past the knots, the unit was reweighed and was ready for use. The time from pithing the amphibian to placing the preparation in the cup was usually 9–12 min.

The balance of O_2 uptake and CO_2 output of a tied inside-out tube of acid-secreting mucosa should be similar to that of open sheets of acid secreting tissue, if as much alkali as acid is formed (Fig. 2). In this case, however, it is possible to prove that secretion occurs, by estimating the changes in the HCO_3^- content of the internal and external media.

The lumen of an inside-out gastric mucosa is not big enough to hold sufficient medium to supply the tissue, so a source of nutrient was attached to it. Tiny glass U-tubes were made, and a 4 ml. balloon of very thin rubber tied to one arm. A clean intact mucosa was then turned inside-out by passing fine forceps through the cardiac sphincter, seizing the pyloric end and sliding the tissue along the instrument. After tying the pyloric end, the cardiac end was attached to the other arm of the U-tube. The nutrient solution was injected into the balloon with a hypodermic syringe, and the air inside the preparation completely sucked out. After tying off the puncture hole, to prevent leakage, the preparation was weighed and was then ready for use (Fig. 2).

Manometric procedure. The O_2 uptake, the resultant of O_2 uptake and CO_2 output, and the anaerobic glycolysis were measured with Warburg manometers in the usual way at 40·0° with mammalian, and 25·0° with amphibian tissue. Conical cups (20–30 ml. capacity), with a centre well, were used for most of the experiments. Histamine and other agents were added from the side arms, or from Keilin tubes (Keilin, 1929). The values of the Q_{O_2}, Q_{CO_2} and Q_{HCl} are in μl./mg. dry wt./hr.

Final treatment of tissues. At the end of the incubation, open tissue was washed in distilled water and dried overnight at 110°. Tied bags of mucosa, now distended by secretions, were uniformly blotted, the small blobs of mucus formed at the rosette ends removed, and the residue hung on the torsion balance by the silk loop and weighed. The contents were drained into a small sample tube, after cutting open the swollen bag of mucosa, which was reweighed when empty. The silk was removed and the tissue washed and dried as before. From the various weights, both the amount of secretion and the total content of the isolated mucosa can be estimated.

When the tissue had been used inside-out, care was taken to drain off as much as possible of the external fluid, whose volume was measured after transference to a 5 ml. graduated tube. The balloon, glass tube and mucosa were then blotted, weighed, and the internal fluid drained as completely as possible into a 5 ml. graduated tube. The volume of liquid transported by the mucosa could thus be calculated. The tissue was cut off, washed and dried as before.

Estimation of the amount of acid secreted. A sample (10–100 mg.) of the gastric contents was weighed into the side arm of a Warburg cup, and, after equilibration at 25° with 5% CO_2 + 95% O_2, was tipped into 4 ml. of a solution containing 0·02 M-$NaHCO_3$ and 0·1 M-NaCl. This solution is isotonic with frog serum and secretion, so no gas exchanges due to differences in vapour pressure occur on mixing. From the output of CO_2 the acidity of the gastric contents can be calculated to an end point of pH 7·4. This pH was chosen because the secretion was ultimately formed from the medium, which had a pH of 7·4.

Bicarbonate estimation. After removing the tissue, a sample of the medium was placed in the main compartment of a Warburg cup and equilibrated at 25° with 5% CO_2 + 95% O_2. 0·1 ml. N-HCl per ml. of medium was then added from the side arm. The output of CO_2 was compared with that from a sample of the original medium, to determine the change in bicarbonate concentration. Since CO_2 is less soluble in acid than in neutral solutions (Dixon, 1943), this technique does not give directly the absolute quantity of bicarbonate in either solution, but gives a sufficiently accurate value of the small difference between the two solutions.

Lactic acid was determined by the method of Friedemann & Graeser (1933).

RESULTS

A. *The metabolism of sheets of gastric mucosa*

Sheep gastric mucosa. It is necessary to know whether the tissue is sufficiently thin to allow adequate amounts of O_2 to diffuse into it to maintain its metabolic activity. The thickness of samples of sheep abomasal mucosa, determined from the weight of a known area, was from 0·3 to 0·6 mm., average 0·5 mm., assuming a density of 1. The ratio dry weight/wet weight was 0·2. The formula of Warburg (1923), which is of doubtful validity when applied to a tissue such as gastric mucosa, which contains cells of differing metabolic activities, gives the upper limiting value of the $|Q_{O_2}|$ as 16 at 38° at which the O_2 pressure at the centre of the tissue is just zero. The Q_{O_2} was measured in 56 out of 127 experiments, and was between $-2·9$ and $-7·0$, average $-4·7$, standard deviation 0·81. Sheep mucosa thus appeared to be a satisfactory tissue for the study of HCl secretion.

When the tissue was incubated aerobically in Warburg cups, the Q_{O_2} and Q_{CO_2} had the usual magnitudes in bicarbonate or phosphate salines or serum, and there was a slight excess CO_2 evolution which corresponded with the lactate formed in the medium. No excess CO_2 evolution due to HCl reacting with HCO_3^- in the media was detected, even after the addition of histamine to concentrations from 5×10^{-8} to 5×10^{-4}M, though in the middle of this range the Q_{O_2} was usually increased by 5–20%. Variations in the tissue/medium ratio had no effect, nor did the addition of extracts of gastric mucosa, so failure to detect acid secretion did not appear to be caused by a loss of diffusible coenzymes.

Various compounds, which might be expected to take part in mechanisms of type 1, were added to discover their effect on the Q_{O_2}. Acetyl choline and eserine, at concentrations from 5×10^{-7} to 5×10^{-4}M, did not alter the Q_{CO_2}, whilst 0·005 or 0·0025M-NH_4^+ had no effect and was not utilized, either alone or together with 0·005 or 0·0025M-ornithine, in the presence or absence of histamine or acetyl choline and eserine. 0·0025M-nicotinamide methochloride, either alone or together with 0·0025M-glycocyamine, was without effect on the Q_{O_2} or Q_{CO_2} of sheep abomasal mucosa.

Frog gastric mucosa. Sheets of frog gastric mucosa also failed to show evidence of acid production, but, after the addition of histamine to a concentration of 5×10^{-5}M, the rate of O_2 uptake was usually increased, often by 10–20%, but sometimes by more than 100% (Fig. 3). No change was observed when the balance of O_2 uptake and CO_2 output was measured (Fig. 4). This means that if HCl was produced an equivalent amount of alkali was also formed.

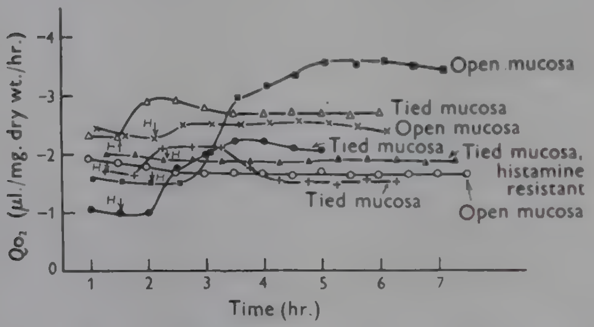

Fig. 3. Typical response curves of seven frog gastric mucosae. Open sheets and tied tubes of mucosa incubated in nutrient phosphate saline gassed with O_2 at 25·0°. Histamine added in six cases to a concentration of 5×10^{-5}M at the arrows marked *H*.

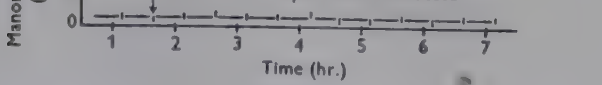

Fig. 4. The balance of O_2 uptake and CO_2 output in five frog gastric mucosae. Preparations incubated at 25·0° in nutrient bicarbonate saline gassed with 5% CO_2 + 95% O_2. Histamine added in three cases to a concentration of 5×10^{-5}M at the arrows marked *H*. The vertical lines represent the readings and their probable error.

When a complete gastric mucosa was cut across to give samples of tissue from the cardiac, corpus and pyloric regions, which were then incubated, in all of three cases the pyloric mucosa had a low Q_{O_2} and failed to respond to histamine, whilst the other two were more active and responded in accordance with their content of oxyntic cells (Table 1). In all of four cases strips from a single mucosa, obtained by cutting the tissue from pylorus to oesophagus, had similar Q_{O_2} and Q_{CO_2} values to within about 10%. This fact was utilized to compare the O_2 uptake of a strip in phosphate saline with the uptake calculated from the gaseous exchanges of two similar strips from the same mucosa in bicarbonate saline, using the indirect method of Warburg (Dixon, 1943). The use of this indirect method of measuring the Q_{O_2} presupposes that the ratio of tissue to medium has no effect on the O_2 uptake. This was checked by using three strips, cut lengthways from both of two mucosae, in phosphate saline. The tissue/medium ratio was varied by a factor of 7·5, and in both series the basal and histamine-stimulated rates agreed within the normal variation.

Table 1. *Effect of* 5×10^{-5}M-*histamine on* O_2 *uptake of various regions of frog gastric mucosa*

(A. Mucosa cut across into three sheets of approx. equal area, consisting largely of cardia, corpus and pyloric antrum of dry weight 20·1, 16·1 and 9·4 mg. respectively. B. Mucosa cut lengthways into three strips of approx. equal area and dry weight. Incubated at 25·0° in 4 ml. phosphate saline containing 0·2% glucose. Histamine added from side arm after 2 hr. Duration of response at least 5 hr.)

Source of mucosa	Av. Q_{O_2} before histamine (µl./mg. dry wt./hr.)	Max. Q_{O_2} after histamine (µl./mg. dry wt./hr.)
A		
Cardia	−1·4	−2·7
Corpus	−1·5	−3·5
Pyloric antrum	−1·2	−1·3
B		
Greater curvature	−1·0	−2·6
Posterior region	−0·9	−2·5
Anterior region	−0·9	−2·4

After a trial of many temperatures between 5° and 40°, 25° was chosen for experiments with frog gastric mucosa, because it is high enough to allow active metabolism, yet safely below 30°, above which temperature the tissue suffers heat damage.

B. *The production of* HCl *by tied intact tubes of frog gastric mucosa*

The equivalence of the acid and alkali produced. When an intact tube of frog gastric mucosa was tied with silk at both ends and placed in a Warburg cup in bicarbonate saline gassed with 5% CO_2 + 95% O_2, the change in the gas pressure was similar to that with open sheets of tissue. The small negative pressure change was the resultant of O_2 uptake and CO_2 output, and was usually of the order of 1–2 mm. Brodie solution/0·5 hr. The addition of histamine to a concentration of 5×10^{-5} M was usually followed by a large increased uptake of gas (up to 55 mm. Brodie solution/0·5 hr.) (Fig. 5), and the tube of mucosa became distended with secretion containing mucus and HCl. Histamine-resistant mucosae secreted mucus only (Fig. 4, Table 4), and did not have an increased uptake of gas.

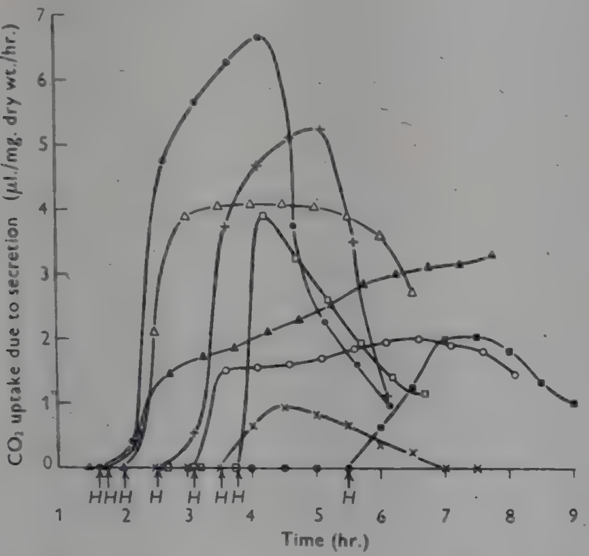

Fig. 5. Typical histamine response curves of eight tied tubes of frog gastric mucosa. Preparations incubated at 25·0° in nutrient bicarbonate saline gassed with 5% CO_2 + 95% O_2. Histamine added in all cases to a concentration of 5×10^{-5} M at the arrows marked *H*.

The following experiments show that this increased gas uptake was due to CO_2 absorption. The experiments on sheets of mucosa showed that the balance of the gas exchanges was not significantly altered by the addition of histamine, and that the loss of HCO_3^- in the medium was accounted for by the amount of lactic acid produced (Table 2). In experiments on acid-secreting closed tubes of mucosa, bicarbonate estimations showed a large increase of this ion in the medium. This increase does not represent the total amount of HCO_3^- formed, because some was decomposed by the lactic acid formed by aerobic glycolysis. The amount of lactic acid in the medium was determined, and a corrected value of the increase of HCO_3^- thus obtained. This value gave good agreement with the extra uptake of gas concomitant with acid secretion, measured in mm. Brodie solution and multiplied by the manometer constant k_{CO_2} for the experimental conditions. This proves that the gas absorbed was CO_2.

The quantity of gas absorbed was also equivalent to the amount of HCl found inside the bag of mucosa (Table 2), and this proves that the mucosa produces

Table 2. *Relation between change in gaseous balance*, HCl, HCO_3^- *and lactate production in typical frog gastric mucosae*

(Open or tied mucosae were placed in 4 ml. nutrient bicarbonate saline and the gaseous balance in 5% CO_2 + 95% O_2 followed at 25·0°. After the experiments the weight of secretions and the amount of acid in the mucosal bag and of HCO_3^- and lactate in the medium were determined.)

	Dry wt. of tissue (mg.)	Wet wt. of secretion (mg.)	HCl produced (µl.)	CO_2 uptake due to secretion (from change in gaseous balance) (µl.)	ΔHCO_3^- in medium (µl.)	Lactate formed (µl.)	ΔHCO_3^- + lactate (µl.)	HCl $-(\Delta HCO_3^-$ + lactate) (µl.)	Duration of experiment (hr.)	Time of addition of histamine (hr.)
A. Histamine to 5×10^{-5} M added during experiment										
Tied	12·6	86±1	+183±2	+180±2	+154±6	+38±4	+192±10	−9±12	5½	1½
Tied	13·8	32±1	+ 41±2	+ 46±2	+ 13±6	+23±4	+ 36±10	+5±12	4	1
Open	15·5	—	—	0±1	− 24±6	+25±4	+ 1±10	—	6	1½
B. No histamine added										
Tied	20·0	11	0±1	0±1	− 33±6	+28±4	− 5±10	+5±11	6½	—
Open	23·9	—	—	0±1	− 40±6	+33±4	− 7±10	—	5	—

alkali, which is neutralized by CO_2 at the same rate as it secretes acid. The changes in the rate of gaseous uptake can therefore be used to follow the changes in the rate of HCl secretion during the experiment. It should be pointed out that, if the ratio volume of fluid (V_F)/volume of gas (V_G) is made large, then the change in the metabolic rate of the tissue after stimulation with histamine can cause an appreciable change in the now large resultant balance of the gas exchanges, which does not occur if the ratio V_F/V_G is small, as in the experiments described (cf. Warburg, 1926).

The relationship between the $Q_{\mathrm{O_2}}$ *and the* Q_{HCl}. In order to correlate HCl production with O_2 uptake, experiments were carried out in phosphate saline gassed with O_2, with alkali in the centre well of the Warburg cup. The increase in the $Q_{\mathrm{O_2}}$ following the addition of histamine (Fig. 3) was associated with acid production, and was less for small than for high rates of secretion (Table 4). In solutions such as phosphate saline and blood, which contain buffers other than the H_2CO_3:HCO_3^- system, retention occurs so that, although the mucosa still produces as much alkali as acid, the HCO_3^- formed reacts with these buffers when it is secreted into the medium. Experiments using frog serum showed that the metabolic activities of frog gastric mucosa were indistinguishable in this medium from those in nutrient saline solutions.

From a knowledge of the magnitudes of the $Q_{\mathrm{O_2}}$ and Q_{HCl} during secretion it is possible to assess some theories of the elaboration of HCl by gastric mucosa. A difficulty is that when the variations in O_2 uptake in a Warburg cup are measured, only the average Q_{HCl} can be estimated from the total amount of HCl produced by the mucosa, and this is less than the maximum value. If the variations in the gaseous balance are followed, the $Q_{\mathrm{O_2}}$ of the tissue is not known, and the regional differences in the tissue preclude the use of the material clipped off outside the knots to find the $Q_{\mathrm{O_2}}$.

The maximum $|Q_{\mathrm{O_2}}|$ of 92 stimulated mucosae was from 1·1 to 4·3, average 2·6, standard deviation 0·56. The maximum $|Q_{\mathrm{O_2}}|$ of 28 sheets of stimulated mucosal tissue was from 1·0 to 3·8, average 2·5, standard deviation 0·57. This shows that tied mucosae were effectively oxygenated by diffusion from one side of the tissue only. The largest frog so far used weighed 64 g. and its mucosa was 0·29 mm. thick. With $|Q_{\mathrm{O_2}}|$ values <5 this thickness is well within the safety limits given by Warburg's (1923) formula, even if the tube of mucosa is tied. The maximum Q_{HCl} observed in 130 experiments (98 with males) was 8·1 and in 20 cases (16 males) the maximum Q_{HCl} rose to more than 4. No sex difference is thus apparent. Secretion could last for only 1 hr. or for at least as long as 8 hr. but usually stopped 4–6 hr. after the addition of histamine (Fig. 5).

All the mucosae with high average rates of secretion were in bicarbonate saline, and it is therefore important to know the $Q_{\mathrm{O_2}}$ of secreting tied mucosae in this medium. The ordinary way of calculating the results, using the second method of Dickens & Šimer (Dixon, 1943), gives an apparent negative glycolysis with secreting tied mucosae. Since with open tissue the amount of glycolysis and the lactic acid found in the medium are in agreement, the true glycolysis in tied tissue can be found by estimating the lactic acid formed, and thus the Q_{alkali} of the mucosa can be calculated. As previous experiments have shown, this is equal to the Q_{HCl}. The disadvantage of this method is that only the overall rates of O_2 uptake, respiratory CO_2 output and glycolysis can be measured, though an estimate of the variations in the Q_{HCl} can be obtained from the changes in the gaseous uptake after histamine stimulation. The mean average $Q_{\mathrm{O_2}}$ in 20 experiments in bicarbonate saline was $-2{\cdot}6$ and in four cases the average Q_{HCl} over the whole period of the experiment was greater than the average $|Q_{\mathrm{O_2}}|$. From the normal shapes of the response curves (Figs. 3, 5) it seems probable that in these experiments the maximum Q_{HCl} was much higher than the maximum $|Q_{\mathrm{O_2}}|$. In any case, only that part of the tissue between the silk loops produced the acid found inside the mucosal bag, whilst the whole unit respired. No account has been taken of this in the calculations, so the evidence is overwhelming that the Q_{HCl} can be higher than the $|Q_{\mathrm{O_2}}|$ in isolated frog gastric mucosa provided with external supplies of CO_2. Several toads have been used, and mucosae from this amphibian behaved in every way similarly to frog mucosae.

The response to histamine. Histamine was more effective at 5×10^{-5}M than in more dilute solutions such as 10^{-6} or 10^{-7}M. This concentration of 5×10^{-5}M is relatively high, but, since the gastric mucosa removes histamine and is relatively thick compared with the distance from the blood capillaries to the oxyntic cells, diffusion will be slow and the actual concentration of histamine in the central layers of the tissue, which contain the oxyntic cells, will be much smaller than 5×10^{-5}M, so the necessity for this high concentration in the medium for optimal results may not be unexpected.

Some mucosae failed to secrete HCl even after the addition of histamine to a concentration of 5×10^{-3}M. The fraction of histamine-resistant mucosae was lower in autumn 1945 and spring 1946 than during the winter; spontaneous secretion appears to be largely a summer phenomenon and did not occur during the investigations described in this paper.

Experiments with 'inside-out' mucosa. Tubes of mucosa turned inside-out, and attached to a supply of nutrient saline in small balloons (Fig. 2), were placed in nutrient saline in a Warburg cup, and the gaseous balance followed in four cases whilst the O_2 uptake was observed in another four experiments. The manometric readings obtained with the inside-out tissue were the same as those obtained with open sheets, both before and after the addition of histamine. This is to be expected only if no secretion occurred, or if the acid secreted into the external fluid liberated a quantity of CO_2 equivalent to that diffusing through the mucosa to neutralize the alkali formed concomitantly with the acid (Fig. 2). Proof that histamine is effective from the acid-secreting side of the mucosa, and that secretion did take place, was obtained by estimating the change in liquid volume and $[HCO_3^-]$ in the internal and external fluids. These estimations show that the acid and alkali are equivalent, with a possible error of about 10% (Table 3).

Table 3. *Secretion by mucosa turned inside-out*

(Inside-out frog gastric mucosae tied at pyloric end and attached by bent glass tubes to thin balloons containing 2 ml. nutrient bicarbonate saline. The units then placed in 2 ml. nutrient bicarbonate saline and the gaseous balance in 5% CO_2 + 95% O_2 followed at 25·0° before and after addition of histamine to a concentration of 5×10^{-5} M at 2 hr. The experiments stopped at 4 hr. and estimations of HCO_3^- and lactate made on internal and external solutions. Typical results.)

	Exp. 1	Exp. 2
Dry wt. of tissue (mg.)	20·6	34·3
Fluid transport (ml.)	0·250 ± 0·010	0·200 ± 0·010
ΔHCO_3^- internal (μl.)	+61 ± 2	+36 ± 2
ΔHCO_3^- external (μl.)	−73 ± 2	−58 ± 2
Lactate internal (μl.)	+ 8 ± 4	+15 ± 4
Lactate external (μl.)	+ 2 ± 4	+ 5 ± 4
ΔHCO_3^- internal due to secretory activity (μl.)	+69 ± 6	+51 ± 6
ΔHCO_3^- external due to secretory activity (μl.)	−71 ± 6	−63 ± 6
Change in gaseous balance after histamine (mm. Brodie solution)	0 ± 0·5	0 ± 0·5

The anaerobic metabolism of frog gastric mucosa. Unstimulated or histamine-resistant tied mucosae secreted appreciable amounts of neutral mucus (Table 4). In the absence of O_2 none of four mucosae produced any secretion. The preparations were as flat and empty at the end of the experiment as at the beginning, and the weight was unaltered. The anaerobic glycolysis, average +1·9 μl./mg. dry wt./hr., was quite unaffected by the addition of histamine. Whilst aerobically the tissue could respire and secrete HCl, for some hours at least, in the absence of external supplies of glucose, anaerobically the glycolysis fell off rapidly under these conditions (cf. Lutwak-Mann, 1947).

Table 4. *Amount and concentration of acid in secretion, aerobically and anaerobically*

(Typical results for tied tubes of mucosa placed in 4 ml. of nutrient phosphate saline gassed with O_2, or nutrient bicarbonate saline gassed with 5% CO_2 + 95% O_2 or 5% CO_2 + 95% N_2 at 25·0°. Complete anaerobiosis secured with yellow phosphorus in centre well. The tube weighed before and after incubation and after emptying. The differences between the increase in weight of the tube, the 'secretion' and the actual 'contents' are probably accounted for by extrusion of mucus into the lumen of the mucosa.)

Gas	Wet wt. of tissue (mg.)	Dry wt. of tissue (mg.)	Wet wt. of secretion (mg.)	Wet wt. of contents (mg.)	Consistency of contents	HCl produced (μl.)	H^+ in contents (M)	Average Q_{HCl}	$\Delta \lvert Q_{O_2} \rvert$ due to histamine	Duration of experiment (hr.)	Time of addition of histamine (hr.)
A. Histamine to 5×10^{-5} M added during experiment											
5% CO_2 + 95% O_2	109	18·3	52	60	Clear jelly	72	0·055	0·71	—	7	1½
5% CO_2 + 95% O_2	120	18·2	156	192	Clear liquid	198	0·046	2·0	—	6½	1
5% CO_2 + 95% O_2	148	24·3	198	234	Clear liquid	424	0·081	3·5	—	6	1
5% CO_2 + 95% N_2	101	19·7	1	0	—	0	—	0·00	—	6	2
5% CO_2 + 95% N_2	81	17·5	0	0	—	0	—	0·00	—	4	1
O_2	93	13·3	49	59	Whitish jelly	74	0·056	1·2	0·3	5½	1
O_2	119	16·5	81	111	Whitish jelly	151	0·061	1·8	0·6	6	1
B. No histamine added											
O_2	152	36·6	47	55	Clear jelly	0	0·000	0·0	—	6½	—

1948

DISCUSSION

The equivalence of the acid and alkali produced by oxyntic cells. In 1859 Brücke assumed that if a free acid was liberated in the gastric tubules, an alkaline fluid of corresponding strength must at the same time pass into the blood and lymph systems (Brücke, 1859). Peters & Van Slyke (1931) have reviewed the many investigations since then into the acid-base balance of the blood, which have demonstrated an increase in its HCO_3^- content apparently associated with gastric HCl secretion. Hanke, Johannesen & Hanke (1931) found that the amount of alkali liberated by the stomach was roughly proportional to the acid secreted, and claimed that the amounts were therefore approximately equivalent. This claim was repeated by Hanke (1937) without support from published evidence. Delrue (1930) first obtained HCl secretion *in vitro* using two chambers, filled to the same level, separated by a sheet of frog gastric mucosa. Although he showed that the pH of the solution at the sub-mucosal side of the tissue rose during secretion, he apparently did not realize the significance of his results. Gray, Adkison & Zelle (1940) repeated these experiments and obtained results 'suggesting that alkali was being liberated from the muscularis surface'.

The experiments described in this paper show that no secretion of HCl could be detected manometrically when sheets of gastric mucosa were incubated in Warburg cups. When the secretion was kept enclosed within the intact tied gastric mucosa HCl production was readily observed. This result in itself shows, with a possible error of about 1 %, that 1 mol. of alkali is produced with each mol. of acid, because the HCl produced by a mucosa of dry weight 40 mg. and a Q_{HCl} 5 μl./mg. dry wt./hr. liberates 200 μl./hr. CO_2, and a change of 1 μl./hr. in the overall gaseous balance can be detected with both inside-out and open tissue. As this change does not occur, 200 ± 1 μl./hr. CO_2 must be absorbed to neutralize the alkali. The experimentally observed equivalence of this acid and alkali, in experiments with the tissue tied and inside-out, supports the conclusion that they are exactly equivalent. This equivalence explains the numerous failures to observe the presence of acid in fresh excised gastric mucosae (Hoerr & Bensley, 1936; Linderstrøm-Lang, Holter & Ohlsen, 1935).

The utilization of external supplies of CO_2 *by oxyntic cells.* As the maximum observed Q_{HCl} is about twice the maximum observed $|Q_{O_2}|$, an actively secreting gastric mucosa may produce acid and alkali at such a rate that a supply of CO_2 in excess of its own respiratory output is needed to neutralize the alkali. There are several reports which support this statement. The neglected work of Edkins & Murray (1924) can now be interpreted as showing that added CO_2 disappears entirely from the lumen of a secreting stomach, and reaches the tissue CO_2 pressure in a non-secreting stomach. McIver, Redfield & Benedict (1926) claimed, by showing that the movement of CO_2 into and out of the stomach is governed by the physical laws of diffusion, that they had refuted this work. As no HCl was secreted during their experiments their claim is unjustified. Kurtz & Clark (1946) recently demonstrated an inverse relationship between the amount of HCl secreted and the amount of CO_2 diffusing into the gastric cavity, whilst Teorell (1933) found negative respiratory quotients using secreting cats' stomachs. These results are all explicable if the actively secreting mucosa utilizes external supplies of CO_2.

The rates of respiration and of acid secretion in mammalian gastric mucosa. The result obtained with frog mucosa that the Q_{HCl} can be greater than the $|Q_{O_2}|$ applies also in mammalian mucosa, as can be shown by calculations based on previously published work. Concomitant measurements of the rates of respiration and secretion of a mammalian stomach have been recorded in at least three papers. Using operative procedures which probably damaged the tissue, as judged by the low activities, Teorell (1933) found that 12 g. cat mucosa used 0·45 ml. O_2/min. and produced up to 0·20 ml. 0·1 N-gastric juice/min. The writer has found that the dry weight of cat mucosa is about 20 % of the wet weight. Therefore the Q_{O_2} was -11 and the Q_{HCl} was $+11$. Similar values can be obtained from the data of Ni & Lim (1928) and Hanke (1937) who worked on dogs. Since all the acid was produced by the oxyntic cells, but only a fraction of the O_2 was used by them, the Q_{HCl} of these cells must have been much higher than their $|Q_{O_2}|$. However, the rates of acid secretion by these vivi-perfused stomachs were much lower than those for gastric tissue prepared after less operative interference.

In unpublished experiments Mackay & Davies have obtained 51 mg. HCl/15 min. for 2 hr. from a chloralosed cat with an acid-secreting mucosa of dry weight 2·7 g. In this case the Q_{HCl} was 46. Rehm & Enelow (1945) obtained 16 mg. HCl/10 min. from a 21 sq.cm. sheet of dog gastric mucosa. It can be calculated from the figures of Ni & Lim (1928) that their dog's mucosa was 1·3 mm. thick, and it is unlikely that the mucosa used by Rehm & Enelow (1945) was thicker than 1·5 mm. Using this value and a dry weight/wet weight ratio of 0·2 the Q_{HCl} was 93. These values of the Q_{HCl} are far higher than the $|Q_{O_2}|$ of cat and dog mucosa.

Many figures are available for the peak value of secretory activity in man. Glenn (1946) recorded the results of 450 histamine-stimulated fractional gastric analyses. The mode of peak volume (ml./15 min.) × free acidity (clinical units) was 4000 and the maximum value 16,000. Mrs Bradford and the author have found the dry weight in g. of 11 human acid-secreting mucosae to be from 8·6 to 15·5, average 12·0, standard deviation 2·0. The mode of the maximum Q_{HCl} was therefore 30. If it is assumed that the subject with the highest rate of secretion had an acid-secreting mucosa of even 16 g., the Q_{HCl} must have been 90. Rosenthal & Lasnitzki (1928) found that the Q_{O_2} of 11 samples of isolated, unstimulated, human gastric mucosa lay between -5 and -20. The average was $-9·2$.

Since the R.Q. of any gastric mucosa is unlikely to be much more than 1·0 it is clear that the Q_{HCl} of the oxyntic cells and even of the entire acid-secreting mucosa can rise much higher than both Q_{O_2} and Q_{CO_2} in the frog, cat, dog and man.

Bearing of the relationship between Q_{O_2} and Q_{HCl} on some recent theories of HCl *elaboration.* The findings discussed in the preceding paragraph bear on some recent theories of gastric acid elaboration. Davenport (1943), Bull & Gray (1945) and Conway, Fitzgerald & Walls (1945) have variously suggested that the H^+ ions are produced at the same rate as, or arise from, acids such as pyruvic or carbonic formed during oxidative metabolism within the oxyntic cells. Since 2 mol. of pyruvic acid are formed from 1 mol. of glucose, and 6 mol. of O_2 are required to oxidize the glucose to 6 mol. of CO_2 and 6 mol. of H_2O, in the first case the Q_{HCl} could never be greater than one third of the Q_{O_2} and in the last case the Q_{HCl} could never be greater than the Q_{O_2}. The possibility that the oxyntic cells remove pyruvic acid from the blood, secrete the H^+ ions and metabolize the anions is unlikely, because the cells do not require supplies of this acid in order to produce HCl.

The observed relation between the Q_{HCl} and the Q_{O_2} renders untenable any theory that the H^+ ions are produced by the oxidative degradation of any substrate known to take part in intermediary metabolism. The results of these experiments support the view that the reaction fundamentally concerned in the production of HCl in gastric mucosa is

$$H_2O \rightarrow H^+ + OH^-.$$

The H^+ ions are secreted, and the OH^- ions finally neutralized by CO_2 and passed into the blood or nutrient medium. This is comparable with the membrane hydrolysis theory of Hollander (1943), and explains the CO_2 uptake which occurs during acid secretion.

The function of carbonic anhydrase in oxyntic cells. Davenport (1939) found that oxyntic cells contain a high concentration of carbonic anhydrase, and suggested that this enzyme has an important role in the mechanism of the formation of acid. However, Feldberg, Keilin & Mann (1940), using sulphanilamide, and Davenport (1946*b*), using thiophen-2-sulphonamide, both powerful inhibitors of carbonic anhydrase, failed to inhibit acid secretion. On the basis of these results, and some rough calculations, Davenport (1946*b*) believed that the necessity for the enzyme in the gastric mucosa had not been demonstrated. To do this it must be shown that, under conditions in the cell, the uncatalyzed uptake of CO_2 is slower than the observed rate, and that the amount of enzyme present is sufficient to produce the necessary acceleration of this reaction.

The calculations given in the Appendix show that the uncatalyzed rate of CO_2 uptake, to neutralize alkali within actively acid-secreting oxyntic cells, is too slow by a factor which depends on the extent to which the pH of the cell is alkaline to the equilibrium value. For example, in mammalian cells at a pH difference of 0·01, a magnification of 400-fold is required, whereas at a pH difference of 0·1 this is reduced to 40-fold. On the assumptions mentioned in the Appendix, the amount of carbonic anhydrase present in the oxyntic cells of cats is sufficient to produce a magnification of the order of 15,000-fold. This means that even if 99·7% of the enzyme were inhibited, the residual quantity could still produce a 40-fold magnification of the uncatalyzed CO_2 uptake. This may account for the results of Feldberg *et al.* (1940) and Davenport (1946*b*). A concentration of sulphonamide sufficient to produce 99·7% inhibition of the carbonic anhydrase of the gastric cells would necessarily have inhibited the enzyme of the red cells to a similar degree. As the red cells contain only about one fifth as much carbonic anhydrase per unit volume as the gastric cells, the carbon dioxide transport by the blood should have been so seriously affected as to lead to speedy death of the animal (cf. the calculations of Roughton, 1943). It would therefore seem that the gastric enzyme could not have been sufficiently inhibited in any published experiment.

SUMMARY

1. Intact tied tubes of frog and toad gastric mucosa secreted acid and mucus *in vitro* aerobically, and also produced an equivalent amount of alkali ($\pm < 1\%$), which was neutralized by CO_2 and passed into the nutrient solution as HCO_3^- ions.

2. No evidence of HCl secretion was obtained when sheets of sheep, frog or toad gastric mucosa were incubated in Warburg cups, although their respiration was enhanced by histamine. This is in agreement with the conception that HCl secretion into the lumen of the stomach is linked with an equivalent bicarbonate secretion into the nutrient solution.

3. The Q_{HCl} of active mucosae was often higher than the $|Q_{O_2}|$ or the respiratory Q_{CO_2}, and in these

cases external supplies of CO_2 were needed for the neutralization of alkali.

4. Calculations presented in the Appendix show that the uncatalyzed rate of CO_2 uptake, to neutralize alkali within very active oxyntic cells, is too slow by a factor of the order of 100, and make it probable that the necessary extra uptake can be readily produced by the carbonic anhydrase within oxyntic cells.

5. Anaerobically the tissue secreted neither acid nor mucus.

6. Evidence is presented which disproves the theories of HCl elaboration due to Bull & Gray (1945) and Conway *et al.* (1945).

I am greatly indebted to Mrs N. M. Bradford (née Longmuir) for assistance during part of these investigations, and to Prof. H. A. Krebs, F.R.S., for his encouragement, interest and advice in this study.

Appendix

BY R. E. DAVIES AND F. J. W. ROUGHTON, *Unit for Research in Cell Metabolism (Medical Research Council), Department of Biochemistry, The University, Sheffield, and Department of Colloid Science, The University, Cambridge*

The uncatalyzed rate of uptake of CO_2 *in oxyntic cells.* The value of the fraction, total volume of oxyntic cells/volume of acid secreting mucosa, is needed for the calculations. From the figures of Linderstrøm-Lang *et al.* (1935) it can be estimated that the fraction in pig mucosa, including the muscularis mucosa, is about 10%. Estimations on photographs of sections of human gastric mucosa varied from 8 to 11%, with an average just below 10%. Davenport (1939) found in cats that the average concentration of carbonic anhydrase was 39 Davenport units (E_D)/mg. wet weight in oxyntic cells and 2·7 E_D/mg. wet weight in acid-secreting mucosa. The surface cells contained small amounts, so the required fraction is not greater than 7%.

The following calculations are made for a mammalian mucosa with a Q_{HCl} of 50 µl./mg. dry wt./hr. at 38° containing 10% oxyntic cells, both well within the extremes. The output of CO_2 is given by the equation

$$\frac{d}{dt}[CO_2] = -k_u(1+l[B])\,[CO_2] + k_0(1+l[B])\,[H_2CO_3]$$

(Roughton, 1943)

in solutions with $pH < 8$. $[B]$ is the concentration of the proton acceptor in the buffer and l is its catalytic coefficient. Since $[HPO_4^{=}]$ is probably about 0·002 M inside the oxyntic cell and its l value is 8 at 0°, this term, and a correction for the protein buffers, can be neglected and the equation used in the form

$$\frac{d}{dt}[CO_2] = -k_u[CO_2] + k_0\,\frac{[a_H]\,[HCO_3^-]\,f_{HCO_3}}{K_1}, \qquad (1)$$

where $$K_1 = \frac{[a_H]\,[HCO_3^-]\,f_{HCO_3}}{[H_2CO_3]};$$

a_H = hydrogen ion activity, and

f_{HCO_3} = activity coefficient of HCO_3^- ion.

$[CO_2]$ is unlikely to be much more than 0·0011 M, and Irving (1932) gives the 'total CO_2' in dog fundic mucosa as about 30 ml./100 g. so $[HCO_3^-] = 0\cdot0134$ M.

Taking the ionic strength of blood as 0·13 (Hastings & Sendroy, 1925) and assuming similar conditions within oxyntic cells then

$$\log f_{HCO_3} = -0\cdot5\sqrt{0\cdot13} \text{ (cf. Roughton, 1935)}$$
$$= \bar{1}\cdot820$$

and $f_{HCO_3} = 0\cdot66$.

Roughton (1943) gives $k_0 = 89$ mol./l./sec., $k_u = 0\cdot131$ mol./l./sec., and $pK_1 = 3\cdot47$ at 38° at infinite dilution.

Substituting these values in equation (1), at equilibrium

$$\frac{d}{dt}[CO_2] = 0 = -(0\cdot131\times0\cdot0011) + \left(\frac{89\times a_H\times0\cdot0134\times0\cdot66}{10^{-3\cdot47}}\right),$$

therefore, $pa_H = 7\cdot20_7$.

If the pa_H of the cell is maintained below this value, CO_2 is evolved, whilst above it CO_2 is absorbed.

At 0·01 pa_H units on the alkaline side of equilibrium, application of equation (1) shows that

$$\frac{d}{dt}[CO_2] = -0\cdot03\times10^{-4} \text{ mol./l./sec.}$$

At 0·05 pa_H units above equilibrium,

$$\frac{d}{dt}[CO_2] = -0\cdot16\times10^{-4} \text{ mol./l./sec.}$$

At 0·10 pa_H units above equilibrium,

$$\frac{d}{dt}[CO_2] = -0\cdot30\times10^{-4} \text{ mol./l./sec.}$$

The oxyntic cells of the mucosa considered have a Q_{HCl} and a $Q_{HCO_3^-}$ of 500. Taking the dry weight as 0·2 wet weight, the usual value for mammalian mucosa, the rate of CO_2 uptake needed to produce this bicarbonate $\simeq 12\times10^{-4}$ mol./l./sec. The magnification of the CO_2 uptake required is therefore 400-fold at 0·01 pa_H, 75-fold at 0·05 pa_H, and 40-fold at 0·10 pa_H units on the alkaline side of equilibrium.

When the back reaction is neglected the required rate is 8·5 times the maximum uncatalyzed rate of CO_2 uptake. (In his calculations Davenport (1946*b*) neglected the back reaction and also used the low Q_{HCl} of about 11.)

In these calculations it is assumed that the whole volume of the cell is available to absorb CO_2 and that $[HCO_3^-]$ and $[CO_2]$ remain constant. When the cell is dependent upon a supply of CO_2 from arterial blood, diffusion limitations will reduce the steady state level of $[CO_2]$ below 0·0011 M. Thus these figures for the required magnification are minimal values and show the necessity for carbonic anhydrase in actively secreting oxyntic cells.

In frogs, while the Q_{HCl} values are lower by about 6-fold, the velocity constants for the hydration of CO_2 are lower by about 3·5-fold at 25·0°, so the enzyme is also required in frog gastric mucosa.

The effect of the carbonic anhydrase in oxyntic cells on the CO_2 *uptake.* The enzyme units used by Davenport (1939) were determined by the method of Meldrum & Roughton (1933) except that the temperature was 0° instead of 15°. One Davenport unit (E_D) is the amount of carbonic anhydrase which, when dissolved in 2 ml. of 0·1 M-Na_2HPO_4 in 0·1 M-KH_2PO_4 and mixed with 2 ml. of 0·2 M-$NaHCO_3$ in 0·02 M-NaOH, halves the time for the evolution of the second quarter of the total CO_2. If R = the reciprocal of the time with the catalyst, and R_0 = the reciprocal of the time without the catalyst, then there is one E_D unit in 4 ml. when

$$\frac{R-R_0}{R_0}=1.$$

To calculate the effect of enzymic activities, as expressed in Davenport units, on the rate of CO_2 uptake under the conditions prevailing in the gastric mucosa, it is first necessary to relate the Davenport unit to the more fundamental constants of carbonic anhydrase activity used by Roughton & Booth (1946).

According to these authors

$$R_{eo}=\frac{k_{eo}xE}{x+K_{mo}}, \qquad (2)$$

where R_{eo} = the extra rate of CO_2 output in the presence of the enzyme,

k_{eo} = velocity constant for dissociation of the enzyme-complex $E.H_2CO_3$,

$x=[H_2CO_3]$,

E = enzyme concentration, and

K_{mo} = Michaelis constant for CO_2 output.

Equation (2) is not convenient to work with, in view of the difficulty of calculating x exactly, but it may be transformed into a much more manageable form as follows.

Roughton & Booth (1946) have shown that

$$k_{eo}=k_{eu} \quad \text{and} \quad K_{mo}=K_{mu}/K,$$

where k_{eu} = velocity constant for dissociation of the enzyme-complex $E.CO_2$,

K_{mu} = Michaelis constant for CO_2 uptake = 0·009 M at 0°,

K = equilibrium constant of the reaction

$$CO_2+H_2O \rightleftharpoons H_2CO_3,$$

i.e. $$K=\frac{[CO_2]}{[H_2CO_3]}=c.\ 900 \text{ at } 0°.$$

We then have

$$R_{eo}=\frac{k_{eo}xE}{x+K_{mo}}=\frac{k_{eo}KxE}{Kx+KK_{mo}}=\frac{k_{eu}EC'}{C'+K_{mu}},$$

where $C'=Kx$ = the hypothetical concentration of dissolved CO_2 which would be in equilibrium with the $[H_2CO_3]$ at the stage used in Davenport's determination for the calculation of the enzyme units present.

This hypothetical or virtual $[CO_2]$ can be readily obtained if the CO_2-dissociation curve of the Meldrum-Roughton (M-R) mixture used by Davenport is known. The curve in question gives the relation between $[HCO_3^-]$ and $[CO_2]$ at complete equilibrium. It has been specially determined by us over the temperature range 0–38° (see Fig. 6 for results at 0°), by equilibrating 4 ml. of M-R mixture with CO_2 pressures varying from 0 to 200 mm. Hg in a modified Van Slyke constant-volume gasometric apparatus, and then determining the CO_2 contents of the gas and liquid phases. The usual 50 ml. Van Slyke gas chamber was replaced by a special 65 ml. chamber, which was the size of the boats used by Davenport. A more detailed account of the technique and experiments will be published elsewhere.

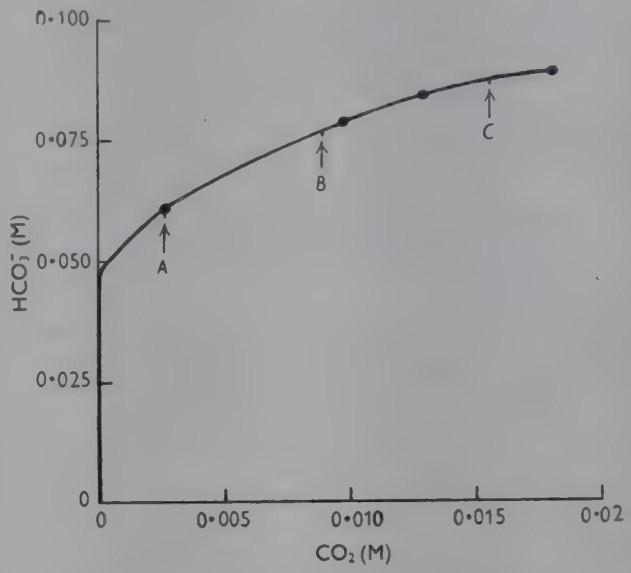

Fig. 6. CO_2 dissociation curve of the Meldrum-Roughton phosphate-bicarbonate mixture. The arrows at A, B and C indicate respectively the conditions at the start, at 3/8 completion and at the end of the reaction, when 4 ml. of M-R solution are shaken in a 65 ml. boat.

By this procedure we found that, at 0°, under the conditions prevailing in the boat experiments,

$[HCO_3^-]$ = 0·0878 M just after mixture,

= 0·0611 M at the end of reaction,

= 0·0778 M when the reaction is 3/8 completed, i.e. at the stage used for calculating the enzyme units present.

At the 3/8 stage, virtual $[CO_2]$ is the abscissa on Fig. 6, corresponding to 0·0778 M-HCO_3^-, and therefore = 0·0094 M.

$[HPO_4^=]$ = 0·07 M and pa_H = 7·24, by calculation.

With Davenport's conditions and 1 E_D enzyme unit in 4 ml., we then have

$$\frac{\left\{\dfrac{k_{eu}E\times 0{\cdot}0094}{(0{\cdot}0094+0{\cdot}009)}+k_u\times 0{\cdot}0094(1+l[HPO_4^=])\right\} - k_u\times 0{\cdot}0094(1+l[HPO_4^=])}{k_u\times 0{\cdot}0094\,(1+l[HPO_4^=])}=1 \text{ at } 0°.$$

Therefore $$\frac{k_{eu}E}{k_u\times 0{\cdot}0184\times 1{\cdot}56}=1.$$

Since $k_u = 0{\cdot}0021$, $k_{eu}E = 6 \times 10^{-5}$ mol. CO_2/l./sec. in the case of 1 Davenport unit.

In cat oxyntic cells there are 39 E_D/mg. wet weight, so 4 ml. of the cells contain $1{\cdot}56 \times 10^5$ E_D units, and therefore in the latter case

$$k_{eu} = 6 \times 10^{-5} \times 1{\cdot}56 \times 10^5$$
$$= 9{\cdot}4 \text{ at } 0^\circ.$$

Within the oxyntic cell, taking $[CO_2]$ as 0·0011 M, and neglecting the catalytic effects of the buffers for reasons given earlier, the magnification of the non-enzymic rate of CO_2 uptake at 0°

$$= \frac{k_{eu}E \times 0{\cdot}0011}{0{\cdot}0011 + K_{mu}} \div k_u \times 0{\cdot}0011$$
$$= \frac{9{\cdot}4}{0{\cdot}0021(0{\cdot}0011 + 0{\cdot}009)} = 440{,}000\text{-fold}.$$

This figure is greater than the magnification of $1{\cdot}56 \times 10^5$ for the M-R solution at 0° because (i) the substrate concentration in the latter case (i.e. 0·0094 M-virtual CO_2) is about the same as the value of the Michaelis constant, whereas in the former case the substrate concentration (i.e. 0·0011 M-CO_2) is only about one-eighth as great; (ii) the oxyntic cells are not known to contain inorganic catalysts corresponding to the $[HPO_4^{=}]$ in the M-R solution.

According to Kiese (1941), and to the rough preliminary data of Meldrum & Roughton (1933), the Michaelis constant increases about 3-fold per 10° rise, and should thus be about 50 to 60 times greater at 38° than at 0°. The substrate concentration, both in the M-R solution and in the case of 0·0011 M-CO_2 solution, is therefore very small compared with the value of the Michaelis constant at 38°; this means, if the effect of $[HPO_4^{=}]$ is neglected, that the magnification must be sensibly the same in the two cases and equal to $k'_{eu}E/k'_u K'_{mu}$, where symbols with primes denote the corresponding constants at 38°.

From these arguments it would follow that

$$\frac{\text{magnification in } 0{\cdot}0011\,\text{M-}CO_2 \text{ at } 0^\circ}{\text{magnification in } 0{\cdot}0011\,\text{M-}CO_2 \text{ at } 38^\circ} = 1{\cdot}8 \times \frac{\text{magnification in M-R solution at } 0^\circ}{\text{magnification in M-R solution at } 38^\circ}.$$

According to the recently amended data of Meldrum & Roughton (Roughton, 1948) on the enzymic and non-enzymic rates of CO_2 output from M-R solutions at temperatures ranging from 0·2° to 34·95°,

$$\frac{\text{magnification in M-R solution at } 0^\circ}{\text{magnification in M-R solution at } 38^\circ} = c.\ 17.$$

Assuming that the value of l for $HPO_4^{=}$ does not alter greatly from 0° to 38° (see below), the magnification in 0·0011 M-CO_2 at 38° should be $1/1{\cdot}8 \times 17 = 1/30$ times the magnification at 0°, so that for the conditions prevailing in the gastric mucosa, the magnification should be 440,000/30, i.e. about 15,000-fold. This figure is only about one fifth of the figure of 70,000-fold at 38° previously given by Davies, Longmuir & Crane (1947). The discrepancy is in part due to the fact that the figure, in the literature at the time, for the effect of temperature on the rate of the enzymic reaction was too high (Roughton, 1943), and in part to a theoretical error in the mode of calculation adopted. It is hoped that the latter is now reliable.

The concentration of carbonic anhydrase in frog gastric mucosa is about 0·1–0·2 E_D/mg. wet weight (Davenport, 1946*a*). Assuming that the concentrations of HCO_3^- and CO_2 are similar to those in the mammalian oxyntic cells, but that the temperature is 25° and the enzyme content 0·15 E_D/mg. wet weight, this concentration of enzyme could produce a magnification of about 1,200-fold.

Since Roughton (1943) states that the phosphate in the Meldrum-Roughton solution and the chloride in the red cell both inhibit carbonic anhydrase to about the same extent at the pH of red cells, no correction need be made for this effect in oxyntic cells.

The uncertainties in these calculations are (1) the extrapolation of the temperature coefficient of the magnification up to 38°, (2) the assumption of a linear relation between enzyme concentration and activity at high concentrations, (3) the possibility that the general environment of the enzyme in the cell may modify its activity, (4) the assumption that diffusion has no limiting effect on the reaction, and (5) the assumption that l is constant from 0° to 38°.

As regards (1) and (5), the uncertainty is probably now not large. In the case of these gastric cells we have no information in regard to (2) and (3), but unpublished calculations by Roughton, based on the rough data of Dirken & Mook (1930) on the rate of CO_2 reactions in red cell suspensions and strong haemoglobin solutions, suggest that the uncertainty may well be only of the order of 2- or 3-fold. The effect of diffusion is very difficult to assess until more exact knowledge is available as to the distances which the CO_2 has to diffuse from the capillaries to the highly concentrated enzyme in the cells.

Apart from the uncertainty as to what limiting effect is caused by diffusion, these calculations make it probable that the necessary extra uptake can be readily produced by the carbonic anhydrase within the oxyntic cells.

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Production of Ulcers in Isolated Frog Gastric Mucosa*

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Previous experiments (see Davies, 1948) have shown that when isolated frog gastric mucosa secretes acid, an equivalent amount of alkali is also produced. Normally this is neutralized by CO_2 and the resulting HCO_3^- ions are passed into the nutrient medium. The amount of HCl produced in highly active mucosae in bicarbonate saline gassed with 5% CO_2 + 95% O_2 (vol. % of gases throughout) was up to 100% above the metabolic CO_2 production and therefore external supplies of CO_2 were needed to neutralize this alkali. During these experiments (done in autumn, winter and early spring) it was found that the more active 'tied bags' of mucosa in phosphate saline gassed with 100% O_2 produced viscous whitish secretions containing much cell debris (indicating tissue damage), although in bicarbonate saline gassed with 5% CO_2 + 95% O_2 the secretions were clear and mobile.

The experiments described in this paper were designed to discover whether ulceration would occur when the experimental conditions interfered with the neutralization of the alkali by CO_2; this would lead to an accumulation of unneutralized alkali.

It was found that whilst mucosae with high secretory activities remained undamaged in bicarbonate saline, active mucosae were regularly ulcerated and even perforated in phosphate salines. Variations in the concentration of the phosphate buffer of the saline medium did not affect the occurrence of this ulceration.

EXPERIMENTAL

Saline media. The bicarbonate saline used for measurements of the balance of the O_2 uptake and the CO_2 output was the physiological salt solution of Krebs & Henseleit (1932) diluted by 0·29 vol. of water and gassed with 5% CO_2 + 95% O_2 or with 5% CO_2 + 95% N_2. During measurements of the O_2 uptake two alternative solutions were used. These were the phosphate saline of Krebs (1933) diluted with 0·29 vol. of water, and a saline solution similar to this phosphate saline, but containing only 0·0011 instead of 0·0084 M-phosphate. Both were gassed with 100% O_2. All salines contained 0·2% glucose.

Material. Frogs (*Rana temporaria temporaria* L.) were captured locally, the web tattooed with Indian ink for identification, and housed in a concrete froggery at 15–17°. An excess of cockroaches or worms was provided for food.

The gastric mucosa was washed, isolated and closed with silk thread at both ends (Davies, 1948). The amount of secretion produced and the total content of the mucosa after the experiment were determined by weighing as previously described. In all cases the preparations were carefully examined for ulcers and perforations. The secretions were classed as clear or cloudy by visual inspection. Microscopical observation showed that cloudiness was due to cells and cell debris.

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* A part of this work was communicated to the Biochemical Society (Davies & Longmuir, 1946).

Manometric procedure. The O_2 uptake, the resultant of O_2 uptake and CO_2 output, and the anaerobic glycolysis at 25·0° were measured with Warburg manometers in the usual way. Conical cups (20–30 ml. capacity) with a centre well were used, and histamine was added from the side arms to a final concentration of 5×10^{-5} M.

Estimation of the amount of acid secreted. In early experiments the previously described manometric method was used (Davies, 1948). In later experiments the acid in samples (10–100 mg.) of the gastric contents was estimated by electrometric titration with carbonate-free 0·01 N-NaOH in the apparatus shown in Fig. 1.

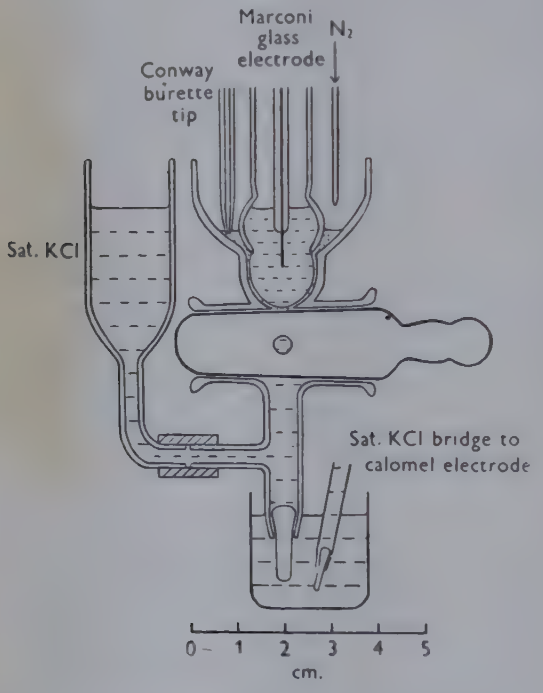


Fig. 1. Semi-micro electrometric titration unit.

A depression in a small glass bowl was ground to fit the thin bulb of a glass electrode (Marconi TM 388 A) so that even 5 mg. of secretion wetted this bulb evenly when it was placed in the depression. The alkali was added from a Conway burette and a jet of N_2 directed on to the surface of the liquid to keep away atmospheric CO_2 and to assist mixing. The electrode was carefully moved up and down after each addition of alkali to ensure complete reaction, which was delayed in many samples by the thick mucus present. A well made tap was fitted just below the depression in the bowl. A saturated solution of KCl leaking upwards round the barrel of this tap at an infinitesimal rate made electrical contact and gave no trouble due to diffusion potentials. A small reservoir of KCl solution maintained the necessary pressure and an internal ground-glass stopper in the tube below the tap prevented leakage downwards. The circuit was completed by a saturated-calomel electrode connected to a Marconi pH meter type TF 511 C by means of a saturated KCl bridge of the type described by Crane, Davies & Longmuir (1948) one end of which dipped into a small beaker of saturated KCl solution which also contained the bottom tube of the titration unit. This apparatus has been used more than 150 times without damage to the electrode and has proved satisfactory and simple in operation.

Units. All Q_{HCl}, Q_{CO_2} and Q_{O_2} values in this paper are in µl./mg. dry wt./hr.

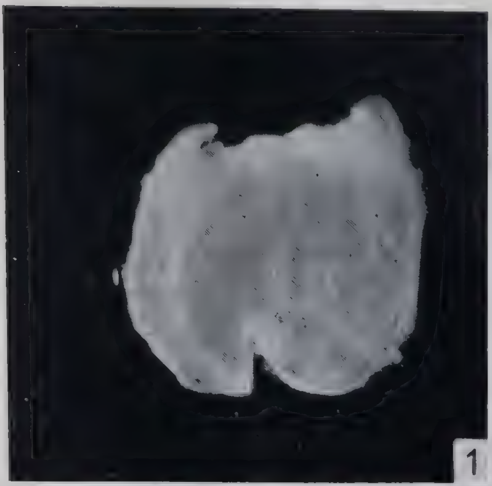
RESULTS

The method of comparing the rates of acid secretion in bicarbonate and in phosphate salines. In previous (winter) experiments (Davies, 1948) spontaneous acid secretion (a seasonal phenomenon) did not occur, and the mucosa produced acid only after stimulation with histamine. In this series of experiments carried out during late spring, summer and early autumn, some mucosae were found to be secreting acid as soon as measurements were taken after equilibration of the cups and manometers. A large overall uptake of CO_2 occurred in experiments in bicarbonate saline gassed with 5% CO_2 + 95% O_2 which, as has been shown previously (Davies, 1948), is indicative of the neutralization of alkali produced by the mucosal bag concomitantly with acid secretion. In experiments in phosphate saline gassed with 100% O_2, with alkali in the centre well of the cup, it was not possible to show that acid had been secreted until the tied mucosa was opened and its contents examined at the end of the incubation period.

Spontaneous acid secretion in bicarbonate saline sometimes occurred not immediately but 1 or 2 hr. after the start of incubation and, since secretion might have begun in experiments in phosphate saline before stimulation with histamine, it is possible to calculate only an upper limit to the average Q_{HCl} of these mucosae during the period of stimulation. Therefore, in order to get comparable figures, the Q_{HCl} was calculated in all cases as an average for the total period of the experiment. Histamine was usually added 1 hr. after the start of incubation which usually lasted 4–6 hr. Frogs were selected at random and, on any given day, alternate mucosae were used in either bicarbonate or phosphate saline.

Experiments in phosphate salines. Table 1 shows clearly that a high average Q_{HCl} was associated with tissue damage and ulcer production in tied mucosae incubated in phosphate salines. Perforations occurred in actively acid-secreting mucosae whether the medium contained a low or a high concentration of phosphate buffer and, as the results were indistinguishable within experimental error, they have been grouped together.

Normal and ulcerated tissues are shown in Pls. 6 and 7. Mucosae with low average rates of acid secretion were not perforated, but the cloudiness of the mucus (Pl. 6) in some cases showed that some



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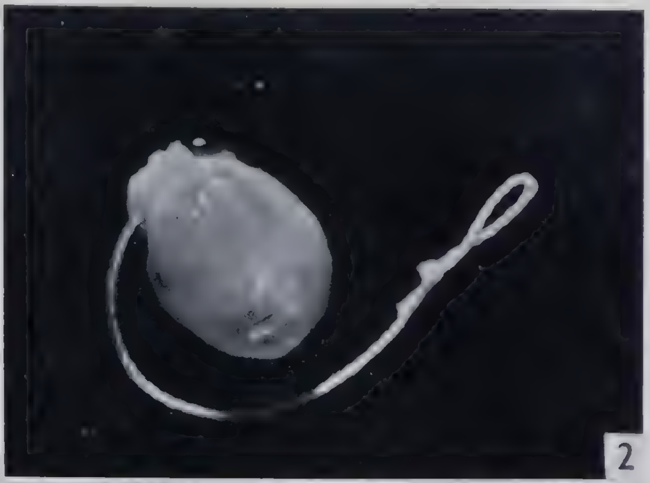

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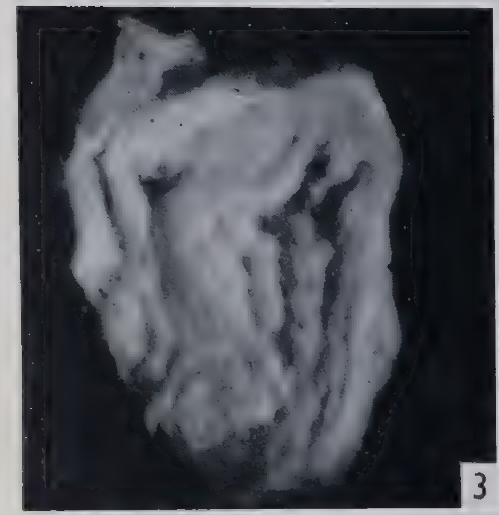

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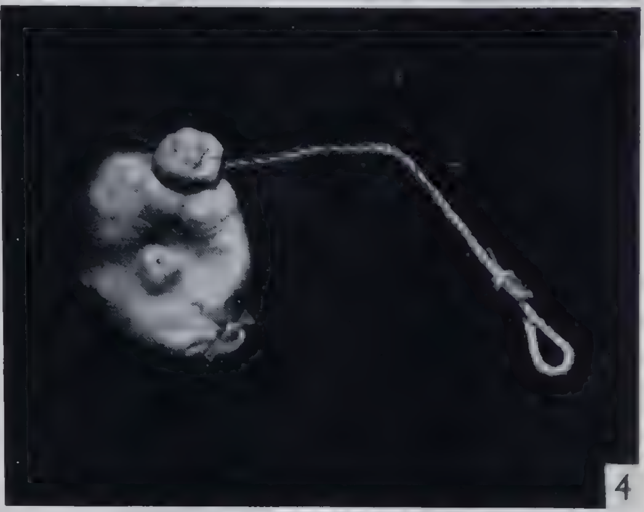

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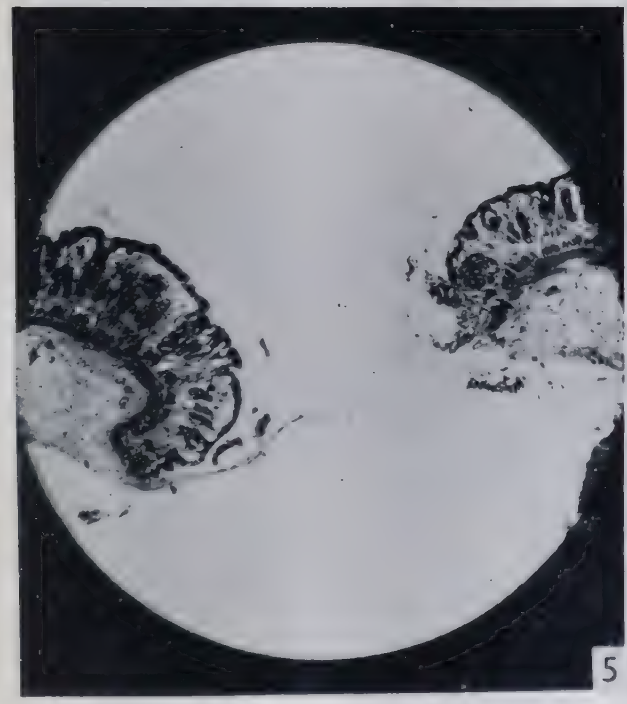

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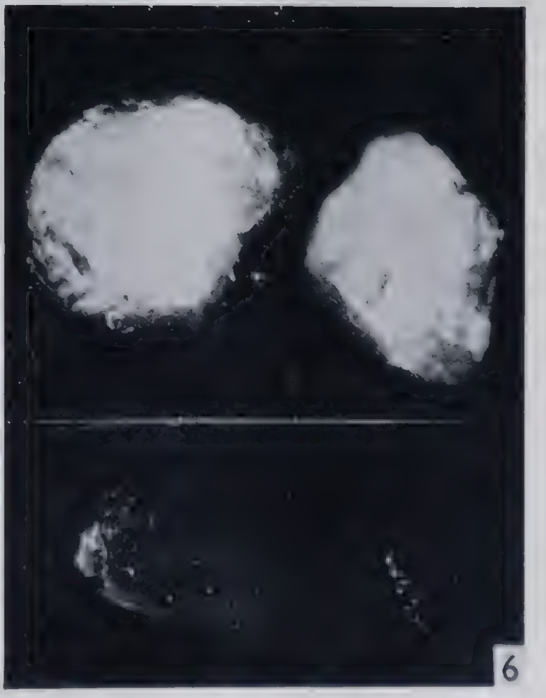

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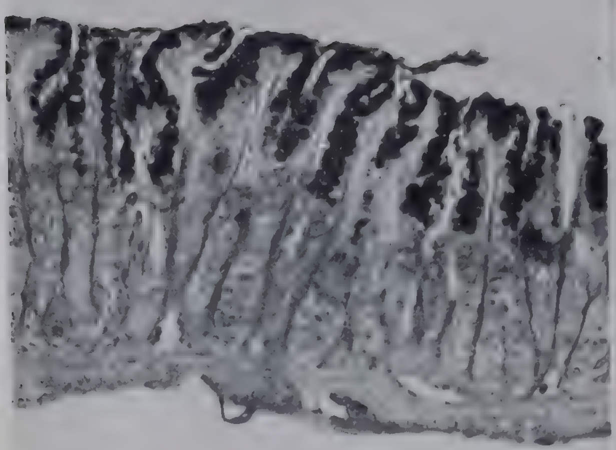
R. E. DAVIES AND N. M. LONGMUIR—PRODUCTION OF ULCERS IN ISOLATED FROG GASTRIC MUCOSA



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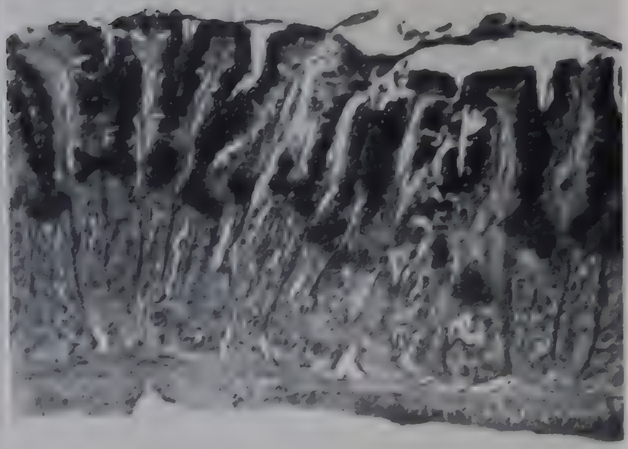

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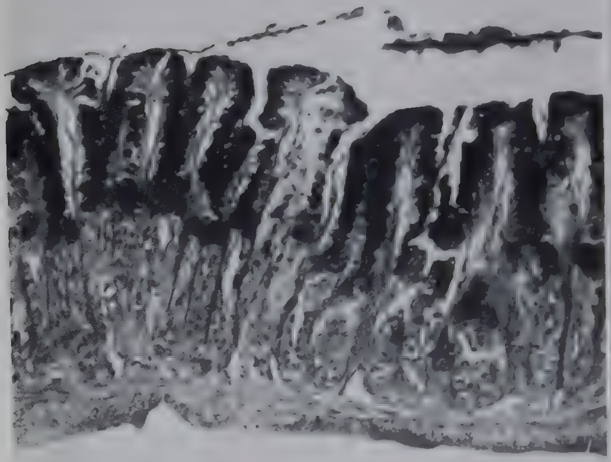

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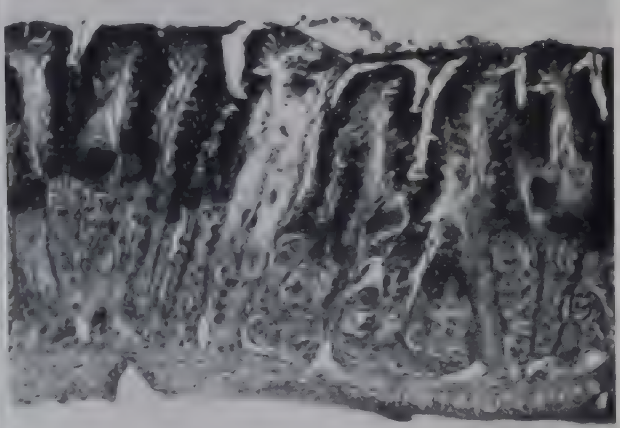

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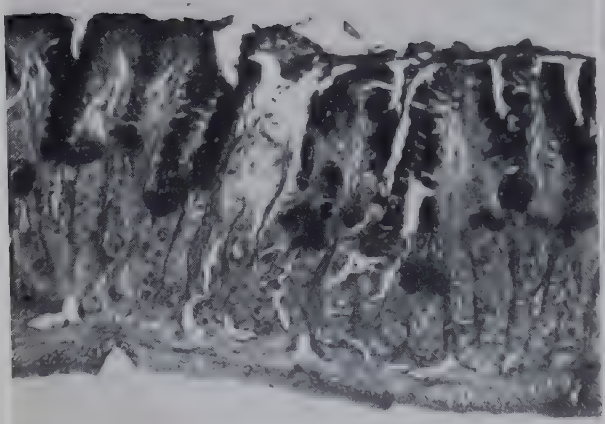

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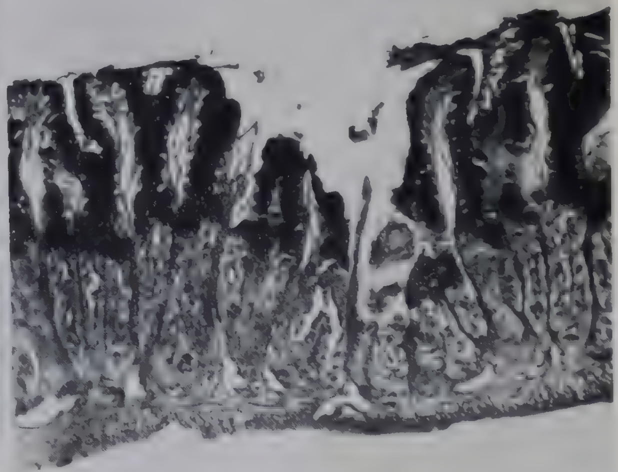

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R. E. DAVIES AND N. M. LONGMUIR—PRODUCTION OF ULCERS IN ISOLATED FROG GASTRIC MUCOSA

Table 1. *Ulcer formation and average Q_{HCl} of tied mucosa in bicarbonate and phosphate salines*

(Total number of experiments 141. Nine tied mucosae under anaerobic conditions produced no acid, no mucus and were not damaged. No damage occurred in open sheets of mucosa in 17 experiments in bicarbonate saline and 15 experiments in phosphate saline.)

	Number of experiments in bicarbonate saline						Number of experiments in phosphate saline					
	No histamine			5×10^{-5} M-histamine			No histamine			5×10^{-5} M-histamine		
Average Q_{HCl} (µl./mg. dry wt./hr.) ...	<0·5	0·5→1·5	>1·5	<0·5	0·5→1·5	>1·5	<0·5	0·5→1·5	>1·5	<0·5	0·5→1·5	>1·5
Clear mucus, no damage	5	1	—	15	8	16	2	—	—	1	7	2
Cloudy mucus, small ulcers	—	—	—	—	—	1	3	1	—	5	9	4
Cloudy mucus, extensive necrosis and perforated ulcer	—	—	—	—	—	—	—	—	—	—	5	15

damage had occurred. Mucosae which secreted no acid were undamaged. The maximum average Q_{HCl} for the whole period of incubation was 2·8, and in no case was the average Q_{HCl} greater than the average $|Q_{O_2}|$ although the maximum Q_{HCl} may have risen above the maximum $|Q_{O_2}|$ in some cases. In the 54 tied mucosae incubated in phosphate salines the maximum $|Q_{O_2}|$ was from 1·7 to 3·9, average 2·7, standard deviation 0·50. All of 15 open sheets of mucosa were undamaged in phosphate saline. The $|Q_{O_2}|$ of some of these increased by 50–100% after the addition of histamine, an effect which, in tied tissue, is always associated with high rates of acid secretion.

Experiments in bicarbonate saline. Open sheets of tissue in bicarbonate saline were also undamaged in all of 17 cases. The results for 46 tied mucosae are shown in Table 1. The average Q_{HCl} had values between 0·0 and 5·4 and the maximum Q_{HCl} values estimated by the method of Davies (1948) were from 0·0 to 7·6. All but one of these mucosae were undamaged and the secretions were clear. The one damaged mucosa had the relatively high average Q_{HCl} of 3·7 and maximum Q_{HCl} of 5·1, and although the central regions of the tissue were undamaged and most of the secretion was clear and mobile, a spot of cloudy, opaque mucus was found in a fold of the tissue at the cardiac end of the mucosa, and beside it was a tiny ulcer.

Experiments under anaerobic conditions. In 9 experiments in bicarbonate saline gassed with 5% $CO_2 + 95\%\ N_2$ the mucosae produced neither acid nor mucus after the addition of histamine and were undamaged.

The secretions of normal and ulcerated tissue. The secretions from ulcerated mucosae were always viscous and opaque and contained much cell debris (Pl. 6) which would be expected to have reacted with some of the acid. Semi-microelectrometric titration of the secretions with 0·01N-NaOH, using the apparatus described (Fig. 1), showed that this was the case. For an equivalent molarity the clear mobile, acid secretions had a lower pH than the cloudy and were relatively less buffered (Figs. 2–4). All neutral secretions were thick with mucus. The cloudy secretions, due to their content of broken-down cells in addition to the mucus normally present, were more strongly buffered (Figs. 5–7). The theoretical curves are given for comparison on the assumption that the secretion was a pure unbuffered HCl solution of equivalent strength titrated to pH 7·4 (the pH of the saline media) with 0·01N-NaOH. Since the acid was usually more concentrated than this alkali, the curve of ml. alkali against pH of reaction mixture shows a point of inflexion between pH 1 and 3. At any pH value, the deviations of the slopes of the experimental and 'unbuffered' curves give a measure of the buffer capacity of the secretions. All the samples were buffered in the alkaline region due to the presence of mucus, but only the secretions containing cell debris from mucosae incubated in phosphate salines were strongly buffered at low pH values.

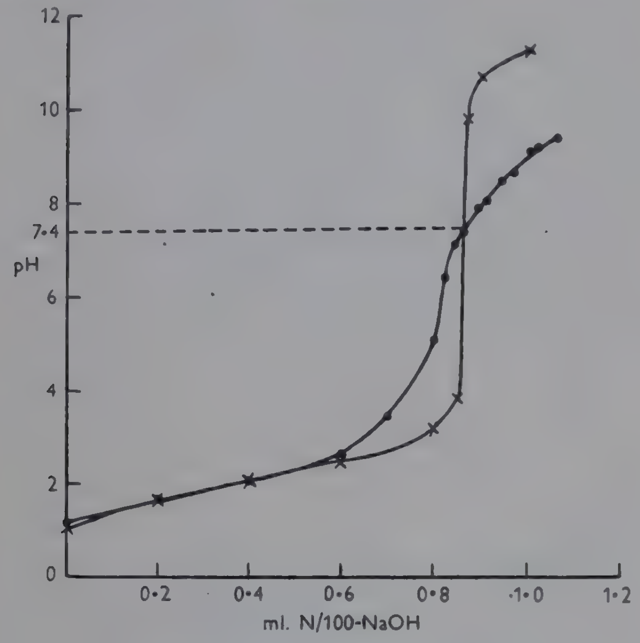

Fig. 2. 100·8 mg. gastric acid.

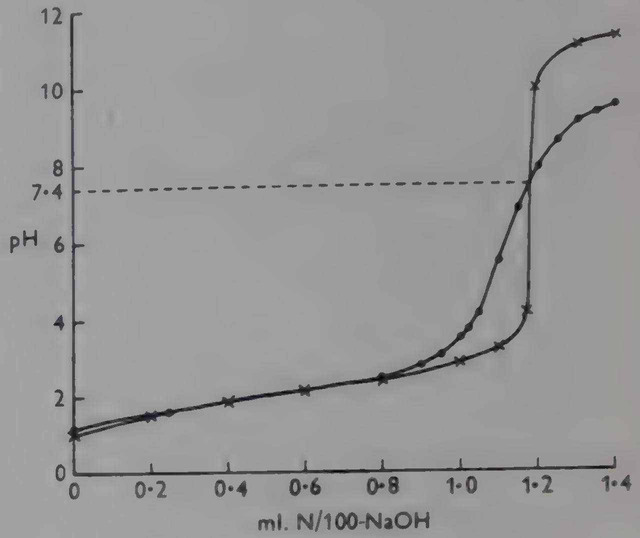

Fig. 3. 118·1 mg. gastric acid.

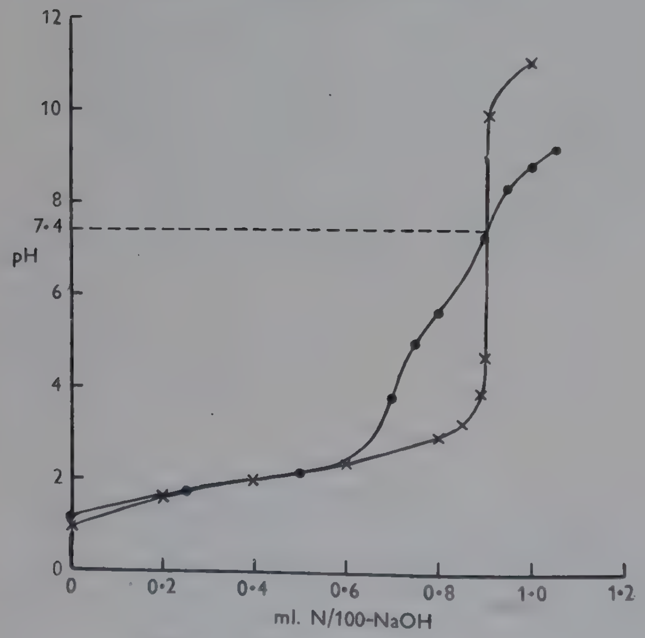

Fig. 4. 86·8 mg. gastric acid.

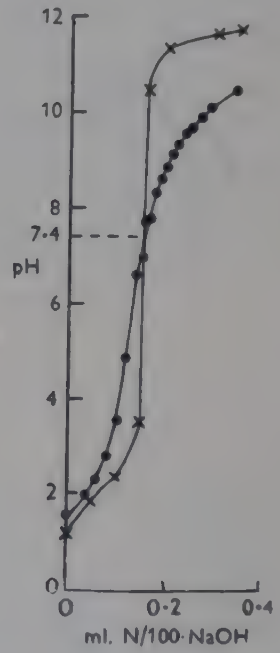

Fig. 5. 20·3 mg. gastric acid.

Figs. 2–7. Electrometric titration (dots) of samples of gastric acid with 0·01 N-NaOH to pH 7·4. The theoretical curves (crosses) were drawn on the assumption that the gastric contents were pure HCl solutions. For further details see Table 2.

DISCUSSION

Ulcer formation in the presence and absence of external supplies of CO_2

The experiments recorded in this paper show that, in the absence of the normal CO_2:HCO_3^- buffer in the medium, extensive tissue damage and perforation can occur in isolated tied bags of frog gastric mucosa with high rates of HCl production. Such damage did not occur in either tied mucosae not secreting acid or with a low Q_{HCl}, or in open sheets of tissue. This could be predicted from previous results (Davies, 1948) which showed that the oxyntic cells of an actively secreting mucosa required amounts of CO_2 in excess of the total metabolic CO_2 production of even the whole of the tissue. If this alkali were not neutralized then local damage would be expected. Tied mucosae in phosphate salines with a Q_{HCl} less than their respiratory Q_{CO_2} would be undamaged if all the CO_2 were available to the oxyntic cells and none absorbed by the alkali in the centre well of the Warburg cup. With a rate of HCl production greater than the rate at which the CO_2 produced by the mucosa could be absorbed by the oxyntic cells, damage must occur if other buffers are ineffective. It is of interest that Delrue (1933) and Gray & Adkison (1941) found lowered rates of acid secretion in isolated frog gastric mucosa when the HCO_3^- ion was omitted from the medium.

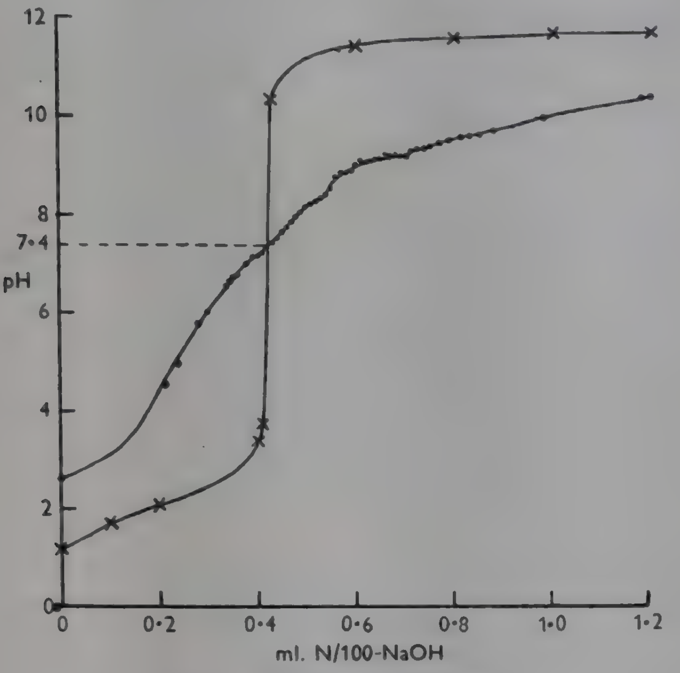

Fig. 6. 62·8 mg. gastric acid.

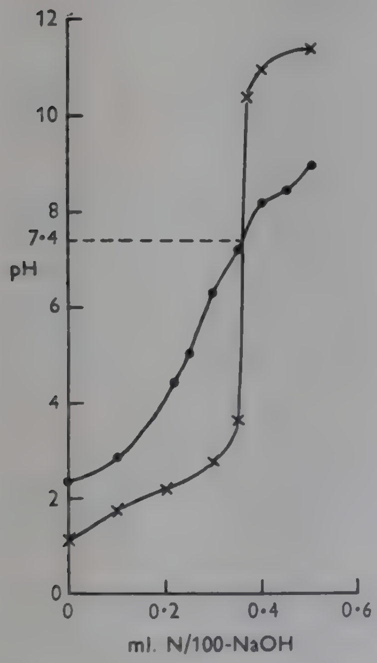

Fig. 7. 47·9 mg. gastric acid.

For reasons given earlier the present techniques preclude an estimate of the maximum Q_{HCl} of tied

Table 2. *Details of the experiments relating to Figs. 2–7*

(Tied tubes of mucosa were placed in 4 ml. nutrient phosphate saline gassed with O_2, or nutrient bicarbonate saline gassed with 5% CO_2 + 95% O_2. The tubes were weighed before and after incubation and after emptying. The differences between the increase in weight of the tube, the 'secretion' and the actual 'contents' are probably accounted for by extrusion of mucus (and cellular debris) into the lumen of the mucosa. Histamine to a final concentration of 5×10^{-5} M was added at the times indicated.)

Corresponding Fig.	Type of saline	Dry wt. of tissue (mg.)	Wet wt. of secretion (mg.)	Wet wt. of contents (mg.)	Consistency of contents	State of mucosa	HCl produced (µl.)	Molarity of H+ in secretion	Molarity of H+ in contents	Av. Q_{HCl} total period (µl./mg. dry wt./hr.)	Max. Q_{HCl} (µl./mg. dry wt./hr.)	Av. Q_{O_2} (µl./mg. dry wt./hr.)	Duration of exp. (hr.)	Time of addition of histamine (hr.)
2	Bicarbonate	39·8	268	283	Clear liquid	Undamaged	544	0·091	0·086	2·7	4·9	—	5·00	1·00
3	Bicarbonate	34·6	173	188	Clear liquid	Undamaged	421	0·109	0·100	2·8	4·5	—	4·20	1·00
4	Bicarbonate	17·9	124	130	Clear liquid	Undamaged	304	0·109	0·104	4·1	6·6	—	4·10	1·00
5	Phosphate	21·5	81	91	Fairly cloudy liquid	1 small ulcer, no perforations	156	0·086	0·077	1·5	—	−1·9	4·50	1·00
6	Phosphate	30·4	149	159	Cloudy liquid	6 small ulcers, no perforations	238	0·071	0·067	1·4	—	−2·3	5·30	1·30
7	Phosphate	25·9	147	174	Whitish viscous paste	1 large perforated ulcer	293	0·089	0·075	1·6	—	−2·1	7·00	1·30

mucosae in phosphate salines. However, the relation between the average Q_{HCl} and tissue damage (Table 1) shows that this damage was associated with high rates of secretion, was less severe with low rates and did not occur in mucosae which failed to secrete acid or gave only slight responses. Despite presumptive evidence of high rates of acid secretion in open sheets of mucosa (large increase in $|Q_{O_2}|$ after addition of histamine) none was damaged. This is because small amounts of CO_2 and HCO_3^- are always present in the phosphate salines during the incubation, and the CO_2 liberated by the acid is thus available to neutralize the alkali in the oxyntic cells and thus reform the HCO_3^- again.

The observation that salines with a high content of phosphate buffer failed to prevent ulceration in actively secreting tied mucosae is evidence that the alkali produced in the oxyntic cells is normally neutralized inside them. This is supported by the known high content of carbonic anhydrase in mammalian oxyntic cells and in frog gastric mucosa (Davenport, 1946), and by calculations (Davies, 1948) showing the necessity for this enzyme in active oxyntic cells. The cytoplasmic buffers may well play an intermediary part in this neutralization, but the overall effect is that the hydroxyl ions are converted quantitatively by CO_2 to bicarbonate and a trace of carbonate at the pH of the medium.

The fact that CO_2 can enter the oxyntic cell without an electric charge, and is then extruded as the negatively charged HCO_3^- ion, may well account for the superiority of the bicarbonate over the phosphate buffer system.

In all but 1 of 46 tied mucosae in bicarbonate saline no damage occurred and the secretions were clear. This mucosa was slightly damaged near a thick fold at the cardiac end. In relation to this case it is of interest to calculate the diffusion limitations on the absorption of CO_2.

Diffusion limitations to the rate of absorption of external supplies of CO_2

Krogh (1919), on the basis of a single determination, found that the diffusion constant of CO_2 was 35·7 times that of O_2 which he gave as 0·14 in muscle at 20°. This is the number of ml. (at 0° and 760 mm.) penetrating through a membrane 0·001 mm. thickness and 1 sq.cm. surface/min. when the pressure difference across the membrane is 1 atm. If the increase of the diffusion constant of CO_2 with temperature is similar to that of O_2 (Krogh, 1919) it will be 5·3 ml. CO_2 (at N.T.P.)/sq.cm./min. at 25° with a pressure gradient of 1 atm./0·001 mm. Warburg (1923) showed that

$$d' = \sqrt{[8C_0 (D/A)]},$$

where C_0 is the pressure of gas outside the tissue in atm., A is the rate of utilization of the gas in ml./min./ml. tissue, D is the diffusion constant of the gas in ml. gas (N.T.P.)/sq.cm./min. with a pressure gradient of 1 atm./cm. and d' is the thickness of the sheet of tissue in cm. at which the gas concentration at the centre is just zero. Applying this formula to the uptake of CO_2 in tied frog gastric mucosa, where the pCO_2 at 5% of 1 atm. pressure can diffuse in from one side only, and taking a dry weight/wet weight ratio of 0·2, we obtain for a tied mucosa 0·03 cm. thick $Q_{CO_2} = -17\cdot5$. If a fold makes the tissue 0·09 cm. thick, then $Q_{CO_2} = -2\cdot0$. These figures give amounts by which the Q_{HCl} can rise above $|Q_{O_2}|$ before diffusion limitations set in and are only approximate as the tissue has been assumed to be uniform. They account for the slight damage occurring at a fold of an active mucosa in bicarbonate saline and show that it is important to tie the silk loops, which close the tube of mucosa at the oesophageal and pyloric ends, at points well away from the corpus of the mucosa to avoid wrinkles in the tissue at the places where the acid is formed and where the CO_2 absorption takes place.

Ulcer formation and treatment

The ulcers shown in Pls. 6 and 7 involve more of the tissue than the oxyntic cells. It seems probable that, once local necrosis has started, the acid already secreted by other cells would extend the damage to other regions of the mucosa. This may be the case in the serial sections in Pl. 7, which show the 'growing point' of an ulcer extending through the tissue.

It is generally agreed that the oesophagus of the frog secretes pepsin, but there has been controversy as to whether the gastric mucosa secretes none at all (Lim, 1922) or small amounts (Friedman, 1937). Since the oesophagus was removed from our mucosae, it seems likely that HCl alone played the most important part in extending the tissue damage.

The significance of these results for the causation of human ulcers is not known. Most chronic human ulcers are formed in regions distant from the site of acid formation (Bockus, 1943), but a combination of factors causing stimulation of active acid secretion, but not providing sufficient CO_2 to neutralize the alkali formed in the oxyntic cells, may be operative in the causation of some types of acute human gastric ulcers. Calculations (Davies, 1948) show that the carbonic anhydrase normally present in oxyntic cells is necessary to facilitate the uptake of CO_2. Therefore, if this enzyme be absent or completely inactivated, tissue damage would be expected to follow on active acid secretion.

Davenport (1946) has recorded the presence of carbonic anhydrase in duodenal mucosa, and it is interesting to speculate whether this tissue acts rather like gastric mucosa in reverse, requiring supplies of CO_2 to secrete $NaHCO_3$ into the lumen of the duodenum and HCl into the blood. If this be true it might throw light on the important experimental results of Cummins, Grossman & Ivy (1947) who found that duodenal ulcers occurred in dogs into whose stomachs physiological concentrations of HCl were dripped. These dogs all developed severe acidosis. However if 0·16N-$NaHCO_3$ was introduced by continuous drip, either by terminal ileostomy or intravenously, in quantities sufficient to maintain a normal blood pH, ulcers did not occur.

The results described in this paper, together with those of Cummins *et al.* (1947), suggest that the ideal antacid for the prevention of ulceration should evolve CO_2 in the presence of acid and should contain cations which do not upset the acid-base balance of the blood. The carbonate or bicarbonate of a basic protein such as a protamine, and milk saturated with CO_2, are therefore put forward as worthy of clinical trial.

SUMMARY

1. In the absence of external supplies of CO_2 (normally required to neutralize the alkali formed concomitantly with the acid), ulcerations and perforations occurred in tied bags of isolated frog gastric mucosa provided that the rate of acid secretion was high. Tied mucosae with low rates of secretion showed little or no ulceration.

2. Tied mucosae which failed to secrete acid, and open sheets of mucosa, suffered no damage.

3. Anaerobically, when mucosae produce neither acid nor mucus, no damage was observed.

4. In the presence of adequate supplies of CO_2, tied mucosae in bicarbonate saline were undamaged.

5. In the absence of external supplies of CO_2 the average Q_{HCl} was never greater than the average $|Q_{O_2}|$. In the presence of 5% CO_2 some mucosae had an average Q_{HCl} greater than the highest $|Q_{O_2}|$ found and a maximum Q_{HCl} about twice the value of this $|Q_{O_2}|$.

6. Ulcers occur if the alkali formed by oxyntic cells remains unneutralized.

The authors wish to record their appreciation to Mr J. Kugler of the Anatomy Department, University of Sheffield, for technical assistance with the histological work and to thank Prof. H. A. Krebs, F.R.S., for his encouragement and interest in this study and Dr H. E. Harding, Senior Lecturer in Pathology, University of Sheffield, for advice on histological and pathological matters.

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EXPLANATION OF PLATES

PLATE 6

Fig. 1. Appearance of normal frog gastric mucosa opened along the greater curvature (cf. ulcerated mucosa, Fig. 3). × 2·3.

Fig. 2. Normal distended tied bag of mucosa after 5 hr. stimulation with 5×10^{-5} M-histamine in bicarbonate saline. The bag was flat and empty at the start of the experiment. Av. Q_{HCl} 3·0, max. Q_{HCl} 4·3. × 2·3.

Fig. 3. Extensive ulcerations in a bag of mucosa (opened along the lesser curvature; cf. Fig. 1). 5 hr. stimulation with 5×10^{-5} M-histamine in phosphate saline. Av. Q_{HCl} 1·9, av. Q_{O_2} −2·6. × 3·7.

Fig. 4. Perforations in mucosa after 5 hr. stimulation with 5×10^{-5} M-histamine in phosphate saline (cf. Fig. 2). Av. Q_{HCl} 1·6, av. Q_{O_2} −2·0. × 2·3.

Fig. 5. Perforation in mucosa after 4 hr. stimulation with 5×10^{-5} M-histamine in phosphate saline containing only 0·0011 M-phosphate. Only a thread of submucosal tissue remains. Av. Q_{HCl} 2·1, av. Q_{O_2} −2·9. 5 µ. section, fixed with ethanolic Bouin, stained with periodic acid and Schiff's reagent (McManus, 1946) and haematoxylin. × 55.

Fig. 6. Samples of clear and cloudy secretions from normal and perforated mucosae respectively. The cloudiness is caused by the high content of cells and cell debris. × 2·3.

PLATE 7

Figs. 7–12. 5 µ. sections of ulcerated mucosa. Figs. 7–11 are serial, but three sections omitted between Figs. 11 and 12. This series shows the extension of ulceration through the tissue. Dissolution of cells starts below the surface epithelium in the distal third of the gland and rapidly involves most of the cells in the distal two thirds. Ulceration occurs a little later by shedding of the surface mucous cells. 5 hr. stimulation with 5×10^{-5} M-histamine in phosphate saline containing only 0·0011 M-phosphate. Av. Q_{HCl} 1·8, av. Q_{O_2} −2·5. Fixed and stained as Pl. 6. × 150.

The Isomeric Transformation of Urea into Ammonium Cyanate in Aqueous Solutions

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Walker & Hambly (1895) showed that the isomeric transformation of ammonium cyanate into urea (Wöhler, 1828) was spontaneously reversible in aqueous solutions. At equilibrium at 100°, a 0·1 M-solution of urea was said to contain approximately 95% urea and 5% ammonium cyanate. Werner (1923) denied formation of cyanate from urea at temperatures below 60°. According to this author aqueous solutions of urea, kept for many months at room or body temperature under sterile conditions, did not contain traces of cyanate.

Since some pharmacological actions of cyanate were recently described (Schütz, 1945, 1946; Birch & Schütz, 1946), the question whether cyanate is formed in the mammalian organism was investigated. As the first step in studying this question, experiments were carried out with the sole aim of establishing whether cyanate was spontaneously formed in aqueous solutions of urea, at body or room temperature. Experiments dealing with living tissues will be reported separately.

The isomeric change in question could, theoretically, be demonstrated by showing a gradual decrease in the amount of urea originally present in the solution. Since, however, even at high temperatures, only a small percentage of the urea originally present is known to undergo the isomeric change into NH_4CNO, the expected decrease would probably be within the margin of error of the most sensitive quantitative methods for the determination of urea. On the other hand, the existing methods for the detection and quantitative determination of cyanate (Walker & Hambly, 1895; Hertig, 1901) were found to be too insensitive, especially when urea was present in higher concentrations.

In this paper two new methods are described for the qualitative and quantitative determination of cyanate in aqueous solutions. By means of these procedures evidence was obtained for the spontaneous formation of cyanate in aqueous solutions of urea, at body and room temperature.

METHODS AND EXPERIMENTAL

SPECTROSCOPIC METHOD

Materials

Methaemoglobin. Human red cells from citrated blood were washed three times with 0·9% (w/v) NaCl solution. The white cells were removed and the red cells were lysed by adding 6 volumes of distilled water. 5% (v/v) of a freshly prepared saturated solution of $K_3Fe(CN)_6$ was added. The solution was left standing for 30 min. at room temperature, and was dialyzed overnight against running tap water through parchment paper, to remove surplus reagent. Thereafter the solution was centrifuged and buffered (10% v/v) with 0·1 M-phosphate buffer (pH 7·3). Only fresh solutions were used.

Urea was recrystallized from ethanol. Sterile solutions were made by dissolving the weighed amount in ethanol; the solution was transferred into a sterile flask, closed with cotton wool, and the solvent removed by placing in a dry oven (100°). After cooling to room temperature, the desired amount of distilled water was added, sterile conditions being maintained throughout. The same results concerning the formation of cyanate were obtained, however, if the growth of micro-organisms was inhibited simply by saturating the solutions with chloroform or toluene.

Spectroscopy

A Hartridge reversion spectroscope was used in a dark room. If previously incubated, the solutions were brought to room temperature before the readings. A concentrated solution of methaemoglobin (metHb) was added drop by drop thereafter until the α-band became just clearly visible. Since the shift depended on the ratio CNO-metHb/metHb, care was taken not to add an undue excess of metHb to the urea solutions. For the readings, the solutions were transferred into containers of *c.* 2 cm. width. Samples which contained much carbonate, formed through decomposition of cyanate, were brought to pH 7·4 by a suitable addition of 0·4 M-phosphate buffer.

Cyanate was found to combine readily with metHb in neutral solutions (Schütz, 1945). On saturation with cyanate the α-band of the absorption spectrum of metHb is shifted from 6325 to 6285 A. This shift can only be observed by means of small dispersion spectroscopes. Since freshly prepared solutions of urea did not alter the spectrum of metHb, the method consists in the determination of the position of the α-band after the addition of metHb solution to the urea solution. Details of the reaction of cyanate with metHb, and with other haemoglobin derivatives, will be reported in a future communication. The method described here is not applicable, without alteration, to solutions containing serum albumin or other substances which also combine with metHb.

Experimental

In preliminary experiments it was found that freshly prepared solutions of urea did not cause the slightest shift of the α-band of metHb added as described above. Neither did the buffer solution, nor the chloroform nor toluene added as a bacteriostatic, cause any change in the position of the α-band.

It was found quite unsuitable to incubate the urea solutions with metHb, not only because the latter quickly coagulates, but also on account of its action as a cyanate-fixing substance. If left in the urea solution for hours, it 'traps' cyanate, thus shifting the equilibrium point of the urea $\rightleftharpoons$ NH_4CNO equilibrium in favour of NH_4CNO. The urea solutions were, therefore, incubated, with or without buffer, either sterile or saturated with a bacteriostatic. At intervals, samples were taken, cooled, and readings made immediately after the addition of metHb. Typical results are shown in Table 1.

The figures show that with increasing time, and concentration of urea, the α-band of added metHb is shifted increasingly towards the point characteristic for CNO-metHb. Since the relevant shift was always very clearly observed with pure aqueous solutions of urea after incubation, the shift cannot conceivably be due to any other substance than cyanate. Moreover, when the maximum shift was observed, the α-band was situated as in the case of pure CNO-metHb.

It was also noted that the transformation, especially at the lower temperatures, is a very slow process. At least 6 hr. incubation at 38°, and more at lower temperatures, is needed to show the presence of cyanate. The lowest concentration of cyanate which can be detected in this way was found to be of the order of 2×10^{-4}M. No quantitative assessment, however, was made with this method, since the manometric method, described below, was found to be superior for this purpose.

Since ammonium cyanate is formed from urea, control experiments were made on the possible action of ammonium ions on metHb, and on the reaction of cyanate with metHb. Under the conditions, and for the short periods, of the experiments shown in Table 1, no influence on either was found by ammonium chloride present in concentrations up to 5% of the corresponding concentration of urea. Ammonium ions, in this range of concentration, had no influence on this qualitative method of detecting cyanate if the pH was maintained within the range 7·0–7·4.

Table 1. *The position of the α-band of methaemoglobin immediately after its addition to fresh or incubated aqueous solutions of urea*

(α-Band of metHb, 6325 A.; α-band of cyanate-metHb, 6285 A.)

MetHb (2–3 drops) added at room temperature to	α-Band (average of several readings) (A.)
1·8 M-Urea in sterile water, freshly prepared	6324 (±4)
1·8 M-Urea in sterile water, after 41 hr. at 38°	6285 (±3)
1·8 M-Urea in water, saturated with chloroform, freshly prepared	6325 (±5)
1·8 M-Urea in water, after 41 hr. at 38°	6288 (±3)
1·8 M-Urea in water, saturated with toluene, freshly prepared	6324 (±5)
1·8 M-Urea in water, after 41 hr. at 38°	6289 (±4)
1·8 M-Urea in 0·4 M-phosphate buffer (pH 7·4), sterile, freshly prepared	6323 (±3)
Same solution, after 41 hr. at 38°	6285 (±2)
Same solution, after 41 hr. at room temperature	6318 (±4)
Same solution, after 5 days at room temperature	6308 (±3)
Same solution, after 41 hr. at 4°	6322 (±4)
Same solution, after 5 days at 4°	6322 (±5)
Controls	
Sterile water	6325 (±4)
0·05 M-NaCNO, in water	6285 (±2)
Water saturated with chloroform, after 41 hr. at 38°	6326 (±5)
Water saturated with toluene, after 41 hr. at 38°	6325 (±4)
0·4 M-Phosphate buffer (pH 7·4), sterile	6324 (±4)
0·4 M-Phosphate buffer (pH 7·4), after 42 hr. at 38°	6325 (±3)

MANOMETRIC METHOD

Materials

Urea solutions were freshly prepared for each experiment and saturated with chloroform. Traces of the latter substance did not interfere with the determinations.

Sodium cyanate. This was made from urea according to Bader, Dupré & Schütz (1948). A pure sample of sodium cyanate was provided by Glaxo Laboratories Ltd.

It is known that cyanate decomposes on addition of acids into CO_2 and NH_3. This was studied with the manometric technique of Warburg. The solution of NaCNO was placed in the main compartment; and buffer solutions of different pH in the side arm. After equilibration at room temperature, the buffer was added to the NaCNO solution, and the CO_2 evolved was measured. The results are given in Fig. 1. It can be seen that only at pH <5 does the decomposition of cyanate become obvious; only in very acid solutions does it decompose rapidly. Contrary to the common belief, cyanate was found to be relatively stable in moderately acid solutions (pH >5).

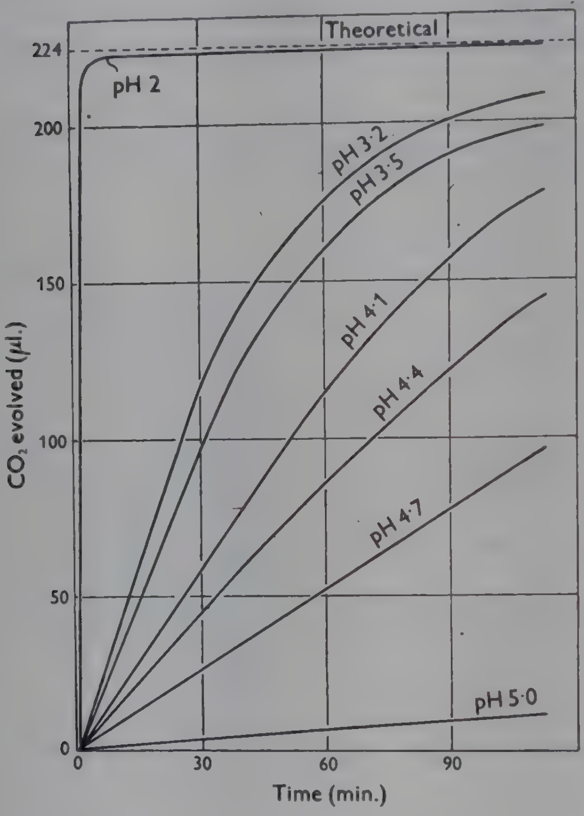

Fig. 1. Decomposition of sodium cyanate in aqueous solution at room temperature and various pH values.

In these experiments NaCNO was preferred to NH_4CNO because of the partial conversion of the latter into urea, even at room temperature. If NH_4Cl was added to NaCNO in equimolar amounts the same results were obtained as with NaCNO alone if the solution was not older than 0·5 hr. Similar results were obtained with different concentrations of NaCNO.

Since cyanate was found to be relatively stable at pH 5·0, it was possible to adopt the following procedure. Any carbonate or bicarbonate present was eliminated by the addition from one side arm of a buffer of pH 5·0. Within the time needed to re-establish equilibrium the decomposition of cyanate was found to be negligible at this pH and at room temperature. Then, after renewed equilibration, an appropriate amount of a strong acid was added from the second side arm, by which the pH of the mixture of cyanate and buffer was brought to 2, or less. The cyanate rapidly decomposed, and the corresponding amount of CO_2 evolved was measured.

Since the amount of CO_2 evolved after addition of the buffer solution (pH 5) corresponds to the carbonate and bicarbonate present in the solution, while that evolved after the addition of the strong acid measured the cyanate present in the mixture, carbonate and cyanate can be determined in the same sample and at the same time. This is useful since cyanate is known to decompose into carbonate to a varying degree according to the prevailing conditions.

Experimental

Incubated samples of solutions of urea were brought to room temperature, and 1–3 ml. of these transferred into the main compartment of a two-armed Warburg vessel, 0·2–0·3 ml. of 3M-acetate buffer being placed in one, and 0·2–0·3 ml. of 10% (w/v) H_2SO_4 in the other side bulb. After equilibration, the buffer was first added and, after re-establishment of equilibrium and noting of the pressure changes, the H_2SO_4 was added from the other bulb, and the CO_2 evolved again recorded.

Typical results are shown in Table 2. With known quantities of NaCNO, the results are in good agreement with the theoretical amounts, in the presence or absence of urea or phosphate buffer or both. It can further be seen that practically no CO_2 was evolved, after addition of the acid to a blank, which consisted of a freshly prepared solution of urea. This indicates that cyanate is not formed rapidly. The small pressure changes observed in this, and buffered blanks, are to be ascribed mainly to the altered CO_2 solubility and the small changes in vapour pressure which occur when the acid is added to the mixture of buffer and urea solution. These blank values are, however, so small in comparison with the data obtained with the same solutions after incubation, that only a very small correction factor would be needed.

Obviously the method is suitable to determine how much cyanate is formed in pure aqueous, or buffered, solutions of urea, since none of the substances present in such solutions interferes with the procedure; neither was any indication obtained of formation of cyanate from urea through manipulations required by the method.

Application of this method to biological material would yield results difficult to interpret, since there might be other substances present, besides cyanate, which would be stable at pH 5 and from which CO_2 would be liberated at pH 2.

With certain limitations, it can be applied, however, to mixtures of biological material and added NaCNO, more concentrated acetate buffer (3M) being required, and a higher concentration of H_2SO_4 to overcome the buffering capacity of biological materials. Results cited in this paper were also obtainable with these higher concentrations of buffer and acid.

Table 2. CO_2 *evolved from solutions after addition of acetate buffer* (pH 5), *showing amount of bicarbonate and carbonate*

(If cyanate is present subsequent addition of H_2SO_4 to the mixture causes a further evolution of CO_2 due to acid decomposition of cyanate.)

	CO_2 evolved (μl.)			
	After 0·3 ml. 3M-acetate buffer (carbonate, etc.)		After further addition of 0·3 ml. 10% H_2SO_4 (cyanate)	
	Found	Theoretical	Found	Theoretical
2M-Urea (fresh), 2 ml.	+ 16	—	+ 3	—
2M-Urea (fresh), 1 ml. 0·1M-Phosphate buffer, pH 7·4, 1 ml.	− 7	—	+ 12	—
0·1M-Phosphate buffer, pH 7·4, 2 ml.	− 7	—	+ 14	—
0·01M-NaCNO, 1 ml.	+ 9	—	+210	+224
0·01M-NaCNO, 1 ml. 0·1M-Phosphate buffer, pH 7·4, 1 ml.	− 6	—	+196	+224
0·01M-NaCNO, 1 ml. 0·1M-Phosphate buffer, pH 7·4, 1 ml. 2M-Urea, 1 ml.	− 13	—	+230	+224
0·01M-NaCNO, 1 ml. 0·01M-Na_2CO_3, 0·5 ml. 0·1M-Phosphate buffer, pH 7·4, 0·5 ml. 2M-Urea, 0·5 ml.	+108	+112	+218	+224

Table 3. CO_2 *evolved from urea solutions incubated for different periods and at different temperatures while saturated with chloroform*

(Carbonate and cyanate determined as in Table 2.)

			'Carbonate' (μl. CO_2)	'Cyanate' (μl. CO_2)	Urea converted (%)
1.	2M-Urea, freshly prepared, 2 ml. 'blank'		+ 16	+ 3	—
2.	1M-Urea, freshly prepared, 2 ml. 'blank'		+ 9	+ 4	—
3.	1M-Urea, 30 min. at 100°, 1 ml.		+ 59	+213	1·21
4.	1M-Urea, 1 day at 4°, 2 ml.		+ 9	+ 6	—
5.	1M-Urea, 2 days at 4°, 2 ml.		+ 4	+ 9	Trace ?
6.	1M-Urea, 4 days at 4°, 2 ml.		+ 4	+ 9	Trace ?
7.	1M-Urea, 2 days at room temperature, 2 ml.		+ 10	+ 10	Trace ?
8.	1M-Urea, 3 days at room temperature, 2 ml.		+ 4	+ 25	0·047
9.	4M-Urea, 24 hr. at 38°, 2 ml.		+ 50	+137	0·104
10.	2M-Urea, 24 hr. at 38°, 2 ml.		+ 31	+ 82	0·126
11.	1M-Urea, 24 hr. at 38°, 2 ml.		− 4	+ 46	0·102
12.	8·30M-Urea	7 days at 38°, 1 ml.	+120	+322	0·242
13.	4·15M-Urea		+ 81	+245	0·359
14.	2·10M-Urea		+ 25	+165	0·412
15.	1·05M-Urea		+ 1	+ 99	0·462
16.	0·50M-Urea		− 9	+ 57	0·508
17.	0·25M-Urea		− 9	+ 42	0·750

In Table 3 representative results are shown, which provide evidence for the formation of cyanate in aqueous solutions of urea at 38°, and even at room temperature. The incubated samples were brought into the Warburg vessels without any further dilution and analyzed as described above. Dilution of the samples after incubation was found to introduce considerable error.

As expected, the formation of cyanate was found to proceed more quickly at 38° than at room temperature. As can be seen from Table 3, comparing Exps. 9–11, with Exps. 13–15, equilibrium at 38° was not obtained within a 24 hr. period of incubation. The amounts found to be formed at room temperature are very small, but are outside the margin of error, since the largest 'blank' ever obtained with pure aqueous solutions of urea, after addition of the acid, was 6 μl.

Similar results were obtained during incubation of urea in presence of 0·1M-phosphate buffer (pH 7·4), 10% (v/v). The formation of cyanate was not greatly altered, but more of it seemed to have decomposed into carbonate in presence of the buffer.

It is obvious that the formation of cyanate proceeds at a very slow rate at these lower temperatures. While at 100° equilibrium is reached within 30 min., several days are necessary at 38°.

Rate of isomerization as a function of concentration. As can be seen from Exps. 12–17 (Table 3) the relative amount of cyanate formed from urea increases rapidly on dilution. This is to be expected, since the thermodynamic equilibrium

$$(NH_2)_2CO \rightleftharpoons NH_4CNO \qquad (1)$$

between an undissociated (urea) and a dissociated component (cyanate) is, on dilution, shifted in favour of the latter; the transformation from right to left (1) is speeded up on concentration.

Both forward and reverse changes, moreover, are slowed down at lower temperatures, but the reaction from left to right (1) a little more so than the reverse; hence the shift of the equilibrium point in 1M-urea solution from 1·21% NH_4CNO at 100° to 0·46% at room temperature.

It will be seen that in Exps. 16 and 17 the values obtained after addition of the buffer solution were negative. This was only observed with samples which had been incubated for many days, and may have been due to the presence of small amounts of ammonia. The negative value (-9 µl.) observed with samples 16 and 17 was taken as zero point, and was added to all the 'carbonate' figures obtained in this series (Exps. 12–17).

The amounts of carbonate formed (corrected) in Exps. 12–17 were added to the amounts of cyanate, since obviously the former was formed by decomposition of the latter. The total was taken to represent the amount of urea converted during incubation. It can be seen that this amount, calculated in percentage of urea initially present, rises rapidly on dilution. This rise becomes steep at lower concentrations (0·005M) which represent the physiological range. The possible physiological significance of this observation will be discussed in a future communication.

SUMMARY

1. The formation of ammonium cyanate in aqueous solutions of urea by isomeric change has been studied by means of two new methods.

2. One method is based on the reaction of cyanate with methaemoglobin, the new compound, cyanate-methaemoglobin, being spectroscopically characteristic.

3. The other method is based on the acid decomposition of cyanate, and manometric measurement of the evolved CO_2; carbonate, present in the same solution, does not interfere, and is determined at the same time.

4. Evidence was obtained with both methods, that, contrary to the opinion of earlier workers, cyanate is formed spontaneously from urea in aqueous solution at 38° and also at 20°.

5. The isomerization is very slow at the lower temperature; equilibrium at 38° is reached after several days. The amounts of urea converted into cyanate at different temperatures, and in different initial concentrations of urea, have been determined.

We are greatly indebted to Prof. H. A. Krebs, F.R.S., University of Sheffield, for helpful advice, and for allowing one of us (F.S.) to learn and use some manometric techniques in his department. One of us (F.S.) is indebted to the Medical Research Council for a grant in aid of the equipment of this laboratory, and to the Mental Disease Research Board of the University of Birmingham for provision of laboratory facilities and grants in aid of this work. We are also indebted to Prof. A. C. Frazer for the help he has given us in presenting the results contained in this paper, and to Dr H. M. Walker, of Glaxo Laboratories Ltd., for presenting us with a sample of pure sodium cyanate.

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The Complexity of the Hyaluronidases Produced by Micro-organisms

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Immunologically distinct hyaluronidases are formed by a variety of micro-organisms when grown in ordinary culture media and are present in large amounts in mammalian testes, leech heads and certain venoms. Because of the complexity and size of the hyaluronate molecule various methods have been used to measure the degree of hydrolysis brought about by these enzymes. Those most frequently employed have utilized viscosity changes or the appearance of reducing sugars in such solutions. Hahn (1945 *a*, *b*; 1946 *a*, *b*), however, has suggested that there are two enzymes present in preparations of testicular hyaluronidase. One of these degrades hyaluronate to a disaccharide and is responsible for lowering the viscosity and for the liberation of about one half of the theoretical amount of reducing sugars; the second hydrolyzes the resulting *N*-acetylhexosamine glucuronide to the constituent monosaccharides. If the ratio of the amounts of these two enzymes varies from preparation to preparation, determinations of hyaluronidase activity by viscosity techniques need not always correlate with those using the liberation of reducing sugars. Other work (Rogers, 1946 *a*, *b*) suggests that the hyaluronidase enzyme system may be even more complex, since, when purified hyaluronic acid is partially hydrolyzed by enzymes prepared from three different sources so that *c*. 50% of the theoretical amount of reducing sugar has been liberated, the size of the units remaining depends upon the source from which the enzyme has been obtained. This observation is best explained (Rogers, 1946 *a*) by assuming that several enzymes successively degrade the polysaccharide to smaller and smaller units; the optimal conditions of pH and salt concentration for the individual enzymes in these systems may differ according to the source of the preparation. Thus, when the hyaluronidases are acting upon the polysaccharide at a single pH and salt concentration, some of the components active in preparations from one source might be inactive in those from another.

If, as McClean (1941*a*) and Lythgoe & Madinaveitia (1943) suggest, the hyaluronidases supply organisms with available carbohydrate by hydrolyzing the hyaluronic acid of the mesodermal tissues, the complexity of the bacterial hyaluronidases has implications for the *in vivo* function of the enzyme, since the organisms will be as dependent upon their ability to form enzymes which liberate fermentable reducing sugars from hyaluronate under physiological conditions as upon their ability to increase skin permeability.

In the present work, the potency of preparations of hyaluronidase obtained from three different bacterial sources has been compared using two different methods of estimation. The first method measures the relatively slight degradation of the polysaccharide (i.e. reduction of the viscosity) and the second, upon a more extensive hydrolysis (i.e. the liberation of reducing sugars). No attempt has been made to separate completely the various enzymes present in any one of the preparations, and no claim is made that the work designates the precise number of enzymes involved in the complete hydrolysis of hyaluronate to monosaccharides. It is thought that claims for specific enzymes, based on the ability of enzyme preparations to hydrolyze *ad hoc* fractions obtained from hyaluronate, such as disaccharides, may be misleading until more knowledge is available of the structure of the complex, undegraded polysaccharide.

METHODS

Viscosity experiments. The technique used was that described by McClean & Hale (1941) as modified by McClean (1943). The final concentration of the phosphate-citrate buffer was M/60 and of NaCl, 0·1 M. In the determination of pH optimum the pH of the final buffer-hyaluronate mixture was always measured because the hyaluronate itself had a considerable buffer action; the glass electrode was used. As in all the previous work, the enzyme potencies (recorded as viscosity reducing units, V.R.U.) are measured by comparison with a dried testicular hyaluronidase preparation of known strength.

Enzyme preparations. The partially purified preparations of streptococcal and staphylococcal hyaluronidase were made by growing a strain of *Streptococcus haemolyticus*, group C (Lancefield) type 7 (Griffiths) (called *Strep.* C7) and *Staphylococcus aureus*, strain Humphrey (called *Staph.* Humphrey) in the casein hydrolysate media already described (Rogers, 1945). The enzymes were partially purified from these cultures by the method appearing under 'partially purified streptococcal hyaluronidase' (Rogers, 1946*a*). Since this latter work appeared it has been found that the value for the ratio of v.r.u./mg. N (i.e. 4550 in Table 1, Rogers, 1946*a*) is too low for the majority of enzymes prepared by this method. When the preparations have been exhaustively dialyzed against a buffer-saline mixture this ratio rises to as high as 66,000 in some instances, and to an average value of 15,000–20,000. It has been found that aqueous solutions of the enzymes are not stable at 0–4° unless protected by the presence of glycerol. Consequently the preparations were dialyzed for about 6 hr. at room temperature against undiluted glycerol and stored at −10°. Under these conditions only a 10–20% loss of enzyme potency has occurred in 9 months.

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Table 1. *The effect of dilution upon a concentrated preparation of* Clostridium welchii *hyaluronidase using various diluents and various periods between diluting and testing*

(The pH of the diluents was always 7·0, temp. 18–20°. The results are expressed as viscosity-reducing units.)

Diluent	Time (min.)	Apparent potency (v.r.u.)
Distilled water	1	227,000
Distilled water	30	116,000
Distilled water	60	100,000
Gelatin (0·2%)	15	650,000
Peptone* (1%)	15	865,000
Gum arabic (0·5%)	2	1,560,000
Gum arabic (0·5%)	30	1,600,000
Gum arabic (0·5%)	60	1,520,000

* As supplied by Evans Sons, Lescher and Webb.

The dilutions made for determination of enzymic activity were in 0·5% (w/v) gum arabic solution. This precaution was necessary since the partially purified enzymes are rapidly inactivated by dilution in water. Table 1, for example, shows the influence of diluting even a relatively crude preparation of *Clostridium welchii* hyaluronidase in a number of diluents. This enzyme preparation was kindly supplied by Dr W. E. van Heyningen, of the Wellcome Physiological Research Laboratories. It was prepared by growing strain S 107 of the organism upon a papain digest medium, and concentrating the enzyme by precipitation with $(NH_4)_2SO_4$ from the culture filtrate. Reducing sugars were estimated by the method described by Somogyi (1937).

Method for estimating the potency of hyaluronidase preparations by the amount of reducing sugar liberated from hyaluronate. No satisfactory test, in which the amount of reducing sugars liberated from potassium hyaluronate is strictly proportional to the concentration of enzyme used, has so far been described. Preliminary tests showed that at pH 7·0 and in the presence of 0·1 M-NaCl the rate at which reducing sugars were liberated by streptococcal hyaluronidase (1–5 v.r.u./ml.) acting at 37° was approximately constant for 30 min. Fig. 1 shows that the enzyme was saturated with substrate under these conditions at a concentration of 0·3 g./100 ml. The sample of hyaluronate used was not pure, and according to the glucosamine determinations on acid hydrolysates, the true concentration was estimated as 0·16 g./100 ml. The method of testing finally adopted was as follows: A solution of crude K hyaluronate (3·3 ml. of 0·5% solution) is mixed with the appropriate (see later) 0·1 M-phosphate buffer containing 0·6 M-NaCl (0·83 ml.) and water (0·37 ml.), and the mixture is warmed at 37° for 1 hr. before the diluted hyaluronidase (0·5 ml. of potency *c.* 10 v.r.u.) is added by carefully running it down the side of the tube to avoid mixing the substrate and enzyme. The tube is corked and the contents mixed at zero time by two inversions. After 22 min. the tube is rapidly cooled in ice water and left for 1 hr. Two samples (each 1·5 ml.) are then taken into a mixture of the Somogyi (1937) $CuSO_4$ reagent (5·0 ml.) and water (3·5 ml.). The reducing sugar is estimated in the usual manner. It is important to boil the tubes to be used in this part of the estimation in chromic acid a few days before use, otherwise the two duplicate estimations do not always agree satisfactorily. The final titrations of the liberated I_2 using 0·005 N-$Na_2S_2O_3$ should agree to within 0·05 ml. for duplicate samples, and usually to within 0·02 ml.

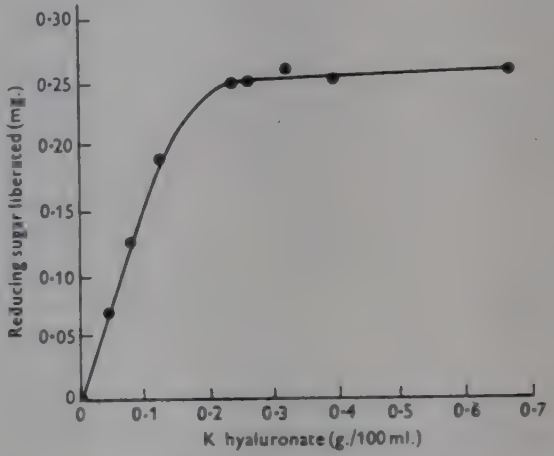

Fig. 1. Influence of substrate concentration on the amount of reducing sugar liberated from potassium hyaluronate by streptococcal hyaluronidase.

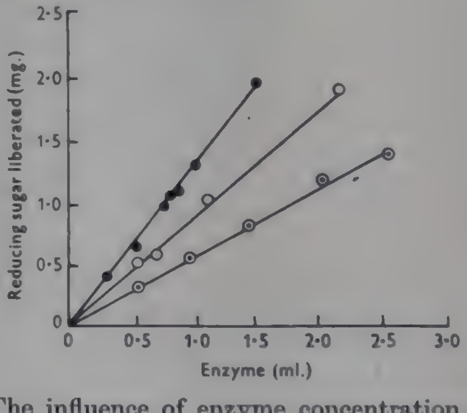

Fig. 2. The influence of enzyme concentration upon the liberation of reducing sugars from hyaluronate by hyaluronidase from *Streptococcus* C7 —●—, *Staphylococcus* Humphrey —○— and *Cl. welchii* —⊙—.

The unit of enzymic activity adopted (r.s.u.) has been arbitrarily defined as the amount of enzyme which will release 1 mg. of reducing sugar, estimated as an equimolar mixture of *N*-acetylglucosamine and glucurone, in 5·0 ml. of the above hydrolysate mixture. Each batch of the $CuSO_4$ reagent must be standardized with an equimolar solution of the *N*-acetylglucosamine and glucurone before use. The same batch of hyaluronate has been used as substrate for all the estimations of enzymic activity by both viscosity reduction and sugar liberation.

Fig. 2 shows that under these conditions the amount of reducing sugar liberated from hyaluronate by hyaluronidases from three different bacterial sources is strictly proportional to the amount of enzyme added.

RESULTS

The pH *optimum of hyaluronidases*

As measured by viscosity reduction. The optimal pH values for the viscosity-reducing activities of testicular and *Cl. welchii* hyaluronidases are 7·0 and 6·0 respectively (McClean, 1943). On the basis of these observations, and because conditions approaching those in the skin of animals were required, McClean (1943) chose pH 7·0 for the standard viscosity reduction assay. Later Hale (1944) showed that streptococcal hyaluronidase could also be estimated viscosimetrically at pH 7·0, thus overcoming the difficulty met by McClean (1941*b*), who found that streptococcal hyaluronidase could not be estimated by the viscosimetric technique of Madinaveitia & Quibell (1940). This failure was due, as Hale (1944) showed, to the rapid inactivation of the enzyme at pH 4·6 which was that originally employed by Madinaveitia & Quibell (1940) and subsequently used by McClean (1941*b*).

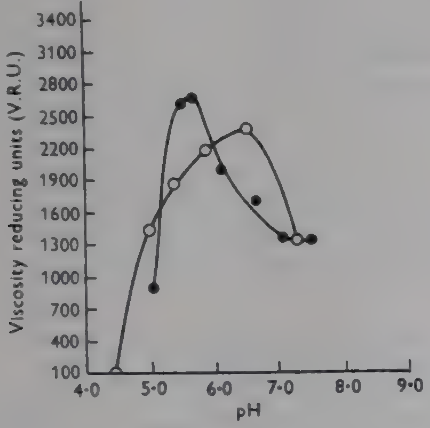

Fig. 3. The influence of pH on the viscosity-reducing activity of hyaluronidase from *Streptococcus* C7 —●— and *Staphylococcus* Humphrey —○—.

Fig. 3 shows the pH optimum of the viscosity-reducing activity of streptococcal (*Strep.* C7) and staphylococcal (*Staph.* Humphrey) hyaluronidase. Both enzymes show a single sharp optimum. The result for the streptococcal enzyme is somewhat unexpected since it shows optimal activity at a pH value of about 5·5, less than 1 unit above that at which inactivation is too rapid for determinations to be made. In this connexion it may be mentioned that the inactivation of this hyaluronidase at pH 4·6 in the presence of substrate is almost completely reversible. After 10 min. at pH 4·6 and 37°, only 4·4% of the original enzymic activity could be detected in a mixture of streptococcal hyaluronidase and substrate. After a further 30 min. the residual value had fallen to 1·0%. At this time the pH was readjusted to 7·0 with 0·5N-NaOH, and an enzyme estimation made immediately showed a 10% recovery of activity, which increased on 3 hr. standing at pH 7·0 and room temperature to 89%. Inactivation of enzyme at pH 4·6 in the absence of substrate could not be reversed in this way.

The staphylococcal enzyme had an optimal pH of about 6·6; this value lies between those for the *Cl. welchii* and testicular enzymes. These curves were constructed using the same buffer system at constant ionic strength. Other types of buffers and other ionic concentrations would probably have altered both the pH optima and the shape of the pH activity curves (cf. McClean, 1943; Madinaveitia & Quibell, 1940; Myrbäck, 1926; Sherman, Caldwell & Adams, 1928).

As measured by release of reducing sugar. Meyer, Hobby, Chaffee & Dawson (1940) and Meyer, Chaffee, Hobby & Dawson (1941) have measured the relations between activity and pH for pneumococcal, *Cl. welchii* and testicular hyaluronidases using the liberation of reducing sugars. Evidence in their paper shows that the amount of reducing sugars, liberated under their conditions, was not proportional to the amount of enzyme used, and consequently their pH optima cannot be accepted without further examination.

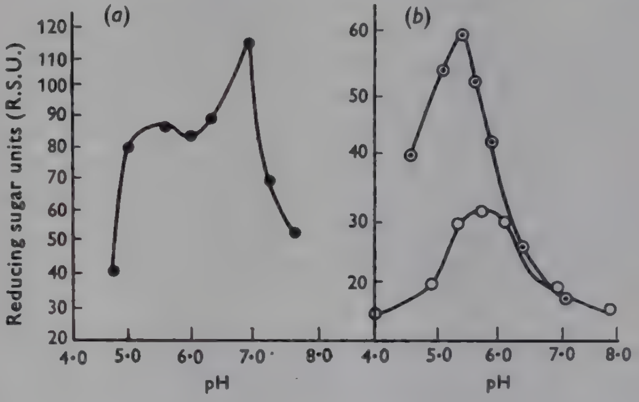

Fig. 4. The influence of pH upon the amount of reducing sugar liberated from potassium hyaluronate by hyaluronidase from (*a*) *Streptococcus* C7; (*b*) *Staphylococcus* Humphrey —○— and *Cl. welchii* S107 —⊙—.

The potencies of preparations of hyaluronidase obtained from *Strep.* C7, *Staph.* Humphrey and *Cl. welchii* strain, S107, were determined at various pH values (glass electrode) by the technique outlined above.

The results are shown in Fig. 4. Noteworthy is the double optimum obtained with the streptococcal enzyme (Fig. 4*a*). Whilst several estimations made with the same preparation were in agreement, the relative values for the maxima were different when a second preparation was examined. This showed a more marked minimum, and the optimum at pH 5·6 represented a higher enzymic activity than that at pH 7·1.

The above result for the streptococcal hyaluronidase suggests that more than one enzyme is involved in the liberation of reducing sugar. It may be significant that one of these optima agrees with the optimum at pH 5·7 obtained by viscosity reduction. A comparison of the pH optima of the other two bacterial enzymes, as measured by the liberation of reducing sugar and by the viscosity reduction technique, shows that only with the *Cl. welchii* preparation is there a close similarity. The optimum pH for reducing-sugar liberation by this enzyme is 5·6, and McClean (1943) obtained the value of 6·0 by viscosity-reduction test. The difference between the optima for the staphylococcal preparation is a whole pH unit.

The ratio of V.R.U./R.S.U. *for different preparations of the same enzymes and for fractions obtained from them*

At this stage in the work a number of glycerinated preparations of staphylococcal and streptococcal hyaluronidase had been accumulated. Some of these had been made from separate batches of enzyme using different seed inocula, whilst others were fractions separated from the same batch during elutions from $Fe(OH)_3$ (Rogers, 1946*a*). The enzymic activities of these preparations were measured by the two techniques at the optimal pH determined above; e.g. the activity of the staphylococcal enzyme was measured at pH 6·6 by the viscosity-reduction technique and at pH 5·5 by the reducing sugar-liberation technique. Table 2 summarizes the results of these tests. There is a large variation between the ratios of the activities estimated by the two techniques, except for those between the two samples of *Cl. welchii* hyaluronidase. The greatest error involved in assessing V.R.U./R.S.U. ratios is ± 15–20 %, being the sum of the maximum experimental errors of the two methods. The difference between the values for the two *Cl. welchii* preparations (i.e. about ± 7 %) is well within the experimental error, and is therefore not significant. These results indicate that the reduction in viscosity, and the liberation of reducing sugars from solutions of hyaluronate, are not brought about by the same enzyme or enzymes in the preparations of streptococcal and staphylococcal hyaluronidase.

Table 2. *Activities of various preparations of bacterial hyaluronidase as measured by the viscosity reduction technique and by the liberation of reducing sugar*

(Both estimations were made at the predetermined optimum pH and with final concentrations of M/60 phosphate-citrate buffer and 0·1 M-NaCl.)

Code number of preparation	Strain of micro-organism	Enzyme potency V.R.U.	Enzyme potency R.S.U.	V.R.U./R.S.U.
C7IV E_1	*Streptococcus haemolyticus* Strain C 7	2,150	17·4*	123
C7IV E_2		1,710	10·2*	169
C7III E_2		2,800	91·0*	30·6
H I	*Staphylococcus aureus* Strain Humphrey	2,620	12·9	203
H Ib		860	6·9	125
H II E_2		2,260	16·2	139
H II E_3		1,610	16·0	100
Pf21	*Cl. welchii* Strain S 107	3,740	19·8	189
Pf21 P_1		15,340	75·9	202

* The liberation of reducing sugar by these samples was measured at pH 7·2, corresponding to the second optimum shown in Fig. 4*a*.

A further separation of the several enzymes was then attempted by chromatography. This method had already given promising results in the purification of *Cl. welchii* hyaluronidase (Rogers, 1946*a*).

Two adsorbents were used: (1) a mixture of equal weights of $Ca_3(PO_4)_2$ and acid-washed powdered glass (pyrex). (2) Al_2O_3 (Savory and Moore Ltd., London). The glycerinated enzyme preparations (5·0 ml.) were dialyzed for 4 hr. against 0·01 M-phosphate-citrate buffer (2 l.) at pH 5·6 and room temperature. It was still necessary to adjust the pH of the dialysates to 5·6 with 0·1 M-citric acid after this period of dialysis. The volumes and the enzyme potencies were then measured, and the dialysates (10 ml.) were passed down the columns of the adsorbents without suction. The sizes of the columns were: $Ca_3(PO_4)_2$-glass powder, 3 × 2 cm.; Al_2O_3, 9 × 2 cm. The columns were washed with water (10 ml.) and then with 0·2 M-Na_2HPO_4 solution. Successive fractions (3–5 ml.) were removed and called E_1, E_2, E_3, etc. These samples were immediately adjusted to pH 7·0 by the careful addition of 0·1 M-citric acid, diluted 1 : 5 in gum arabic (0·5 g./100 ml.) and were stored at −10° until tested. The potencies of the samples were measured by both the viscosity-reduction and reducing sugar-liberation techniques at the appropriate pH.

Again the ratios of the activities measured by the two methods show very considerable variation (Table 3), amounting in some cases to fourfold differences. In almost all cases the variation is at least twice the maximum total experimental error of ± 15–20 %. Similar chromatographic runs were also made with the *Cl. welchii* preparations; there was no significant difference, however, with these enzymes, between the values for the v.r.u./r.s.u. ratios. These results again confirm those obtained for the similar pH optima of the *Cl. welchii* preparations. In Table 3, the differences between the ratios for the various fractions separating from the column of Al_2O_3 are greater than the values for those from the $Ca_3(PO_4)_2$ column, due, presumably, to the more effective separation of the bands of enzyme.

Table 3. *The* v.r.u./r.s.u. *ratio for bacterial hyaluronidase fractions prepared by chromatography*

	Adsorbent $Ca_3(PO_4)_2$ + glass powder		Adsorbent Al_2O_3	
Fraction no.	*Strep.* prep. (v.r.u./r.s.u.)	*Staph.* prep. (v.r.u./r.s.u.)	*Strep.* prep. (v.r.u./r.s.u.)	*Staph.* prep. (v.r.u./r.s.u.)
E_2	50·8*	—	—	—
E_3	79·9*	220	—	—
E_4	91·1*	199	—	—
E_5	—	144	—	—
E_6	—	104	—	—
E_7	—	—	31·0*	89·0
E_8	—	—	133*	59·2
E_9	—	—	67·2*	24·5
E_{10}	—	—	—	—
Original dialysate	93·8	—	80·1	112

* Reducing-sugar liberation by these samples was measured at pH 7·2 corresponding to the second optimum of the curve shown in Fig. 4*a*.

reducing enzyme falls. These considerations suggest that, if there are several enzymes responsible for various stages in the hydrolysis of hyaluronate, there should be considerable variations in the v.r.u./r.s.u. ratios during the course of growth of the organisms.

Two aspects of the problem were investigated: (1) by measuring the v.r.u./r.s.u. ratio in ordinary cultures of organisms at various stages of growth in casein hydrolysate media, both supplemented with hyaluronate (0·5 g./100 ml.) and unsupplemented (20 ml. quantities in pyrex glass tubes incubated at 37°); (2) by measuring the v.r.u./r.s.u. ratio of cultures growing in supplemented medium in cellophan dialysis sacs surrounded by medium containing no polysaccharide. Under these latter conditions it was hoped to prolong considerably the logarithmic phase of growth. The flasks containing the sterile medium and cellophan sacs, mounted on glass tubes in bungs which fitted tightly in the necks of the flasks, were shaken at 37° on an eccentric shaker designed by Duthie (unpublished communication). The shaking helped to stir the contents of the flask and sacs.

In both types of experiment, samples were taken at the intervals indicated in Tables 4 and 5, and centrifuged immediately on an angle centrifuge. The supernatant liquids were drawn off, a drop of toluene added to each, and stored at 0–4° until the end of the experiment. All the samples were then placed in small cellophan sacs and dialyzed for 24–48 hr. at 0–4° against 4–5 l. of 0·01 m-citrate-phosphate buffer, pH 5·8. This dialysis was necessary to remove traces of unfermented glucose and other reducing substances before testing the samples. The viscosity-reduction tests were also made on the dialyzed samples, so that any inactivation of enzymes, which had taken place during dialysis, would be unlikely to upset the evaluation of the v.r.u./r.s.u. ratio.

Table 4 shows the results of two experiments carried out by the first method, in which the v.r.u./r.s.u. ratios were determined after different periods of growth of the *Streptococcus* C7 in the ordinary tube cultures. The figures recorded are the mean of two cultures for Exp. 1 and of three cultures for Exp. 2.

The figures in Table 4 are among the most convincing evidence for the occurrence of differences

Variation in the v.r.u./r.s.u. *ratio with age of the culture*

On examining the values for the v.r.u./r.s.u. ratio of the various preparations of streptococcal hyaluronidase, it was noticed that the largest differences occurred between batches of enzyme for which different seed innocula had been used rather than between different fractions obtained from the same batch. This suggests that the organisms, in different phases of growth and by inherent variation, might produce different amounts of the various enzymes. It is known, for example, that the production of the viscosity-reducing enzyme is adaptively increased by the inclusion of whole hyaluronate (McClean, 1943; Rogers, 1945), or of hyaluronate degraded as far as is possible by testicular hyaluronidase (Rogers, 1945, 1946*a*), but not by the products remaining after it has been hydrolyzed by streptococcal enzyme. Thus it was supposed that, during the growth of streptococci in a medium containing hyaluronate, the level of production of the viscosity-reducing hyaluronidase would be high only so long as there remained an adequate supply of sufficiently large units of hyaluronic acid in the medium to stimulate the organisms. If the formation of enzymes attacking smaller units of the polysaccharide should also be adaptive, then there would be a supply of units of the necessary size in the medium for a somewhat longer period, the large units being progressively degraded by the hyaluronidase formed by the growing organisms. If, on the other hand, the production of these latter types of enzyme were not adaptive, the organism might be expected to go on producing them at a steady level whilst the production of the viscosity-between the values of potency of the enzyme preparations when measured by the two methods; the V.R.U./R.S.U. values show an extreme difference of 12·6–192, giving very satisfactory proof of the complexity of the enzymes hydrolyzing hyaluronate. The hypothetical sequence of events in the formation of enzymes by cultures, as elaborated above, is, however, probably inadequate. An added complication seems to be that some enzyme or enzymes, estimated by the sugar-liberation technique, are sometimes destroyed during the prolonged incubation of the culture.

Table 4. *Values for the ratio* V.R.U./R.S.U. *in the dialyzed supernatant fluids from cultures of* Streptococcus C7

(The organisms were grown for the indicated periods in the casein hydrolysate medium A (Rogers, 1945) supplemented with 0·5 g./100 ml. of a crude sample of potassium hyaluronate, and in the unsupplemented medium. Exps. 1 and 2 were inoculated with different seed cultures of the organism and the results for Exp. 1 are the mean of two duplicate cultures, whilst those for Exp. 2 are the mean of three cultures.)

	V.R.U./R.S.U.		
	+Hyaluronate Exps.		
Time of incubation (hr.)	1	2	No hyaluronate Exp. 2
10	12·6	—	—
24	11·3	21·2	56·2
48	—	60·5	125
96	68·2	94·7	192

Table 5. *The amounts of hyaluronidase formed by* Streptococcus C7 *in tubes and dialysis sacs*

(The organisms were grown at 37° in 25 ml. of the casein hydrolysate medium A (Rogers, 1945) supplemented by 0·5 g./100 ml. of a crude sample of potassium hyaluronate (1) in 'pyrex' glass tubes, and (2) in cellophan dialysis sacs surrounded by 1 l. of unsupplemented medium. The values given are the means for two duplicate cultures.)

Time of incubation (hr.)	V.R.U.		R.S.U.		V.R.U./R.S.U.	
	*D**	*T*†	*D**	*T*†	*D**	*T*†
10	112	62·4	4·13	4·9	27·2	12·0
24	670	221	18·7	19·0	39·4	13·2
48	1250	247	26·2	14·3	47·5	17·2
96	2850	410	26·7	4·7	107	87·0

* *D* = dialysis sac cultures. † *T* = tube cultures.

It was apparent from the results that the adaptive increase of the enzymes, involved in the liberation of reducing sugars from hyaluronate, was at least as great as that of the enzymes estimated in the viscosity-reduction test. It seemed, therefore, of interest to proceed with the experiments in which the organisms were grown in a dialysis sac. In such cultures the smaller saccharide units, formed by the action on the hyaluronate of the hyaluronidase produced by the organisms growing inside the sac, would also be removed. Thus it might be expected that the V.R.U./R.S.U. ratios for the sac cultures would be higher than those for the tube cultures if it is the smaller saccharide units which stimulate the production of enzymes estimated by the R.S.U. technique. The results in Table 5 support this suggestion. The ratios of the V.R.U./R.S.U. values for the sac/tube cultures are 2·3/1, 3·0/1, 2·8/1 and 1·2/1 for the various times at which the cultures were sampled. Apart from the last pair of values, these differences are well removed from the maximum experimental error of ± 20%. A further point of interest emerged from this series of experiments. The V.R.U. titre increased after a period of 96 hr. growth in the dialysis sacs to 2850. Yet, since the formation of hyaluronidase, as assessed by the viscosity-reduction technique in cultures grown in tubes, is strictly proportional to the amount of hyaluronate present in the medium, up to a concentration of 0·6 g./100 ml. (Rogers, 1945), it might be supposed that the concentration of hyaluronate in the medium would be the factor limiting enzyme formation. Apparently, however, when essential metabolites are added and toxic metabolic products removed by dialysis, enzyme production proceeds up to a level of 2850 V.R.U., compared with 250 in the tube cultures. The ratio of V.R.U./mg. bacterial N in the dialysis sac and tube cultures was identical at the end of the experiment. Hence it seems that, once they have begun to produce hyaluronidase at the adapted level, the organisms can continue to do so in the absence of the polysaccharide, providing fresh growth substances are added and toxic catabolites removed. Organisms from dialysis-sac cultures, re-inoculated into fresh medium without hyaluronate, form enzyme only at the unadapted level when the logarithmic phase of growth is resumed.

DISCUSSION

Measurements of streptococcal and staphylococcal hyaluronidases by the methods which depend upon the rate of liberation of reducing sugars do not agree with those which use viscosity-reduction estimations. The differences amount to more than one hundred times the experimental error, and it seems legitimate to conclude that the two methods do not measure the activity of one and the same enzyme. The conditions of buffer and NaCl concentrations were the same in both methods of estimation; therefore the objections raised by McClean & Hale (1941) and by McClean (1943) to the basis for the conclusions reached by Madinaveitia, Todd, Bacharach & Chance (1940) cannot be applied to the present work. The last-named authors thought that the hyaluronidase and diffusing activities of testicular extracts were probably due to the presence of different entities, because of large discrepancies between the potency of the preparations assayed by the viscosity-reducing and skin-diffusion techniques. McClean & Hale (1941) and McClean (1943), however, pointed out that Madinaveitia *et al.* (1940) were measuring hyaluronidase activity by viscosity reduction at pH 4·6 and in M/6 NaCl, conditions far removed from those of the *in vivo* tests in the skin, and showed that the discrepancies were likely to be due to this difference. This is now rather easier to understand, since under the conditions used by Madinaveitia *et al.* (1940) enzymes may be active in reducing viscosity, which would be inactive in the skin of injected animals and in the hyaluronidase assay used by McClean (1943). If this is the true explanation, it might also apply to the results of Humphrey (1946) who found that viscosity reduction and sugar liberation by testicular and *Cl. welchii* hyaluronidase preparations ran parallel, when tests were conducted at a low pH and high ionic concentration.

It is necessary here to consider further the meaning of the variations in the V.R.U./R.S.U. ratios which have been observed in the present paper. The methods of measurement were not designed to determine the precise number of enzymes involved in completely hydrolyzing the complex hyaluronate molecule. First, the extent of the hydrolysis accomplished by the enzyme or enzymes measured in the viscosity reduction technique is unknown. Using a physical measurement such as that of viscosity, hydrolysis can only be followed so long as the molecules are large enough to exert their effect. Consequently we do not know the V.R.U./R.S.U. value characteristic of this stage of hydrolysis, which will presumably be the upper limit for its variation. The highest value which has been obtained in the present work is about 280. Secondly, in a series of enzymes acting successively in the hydrolysis of a high molecular weight substrate, such as is suggested here for the hydrolysis by hyaluronidase of hyaluronic acid, the individual enzymes are not independent in their functioning. Alteration in the concentration of enzymes acting at an early stage in the scheme will alter the rate of action of those coming later. The importance of this type of interrelationship in influencing V.R.U./R.S.U. ratios will depend upon such factors as the number of enzymes involved and their kinetics.

In order to decide how many enzymes are present in hyaluronidase preparations we must know more of the structure of hyaluronate. The most important single contribution would be towards an understanding of the chemical changes underlying the loss of viscosity of solutions of the polysaccharide.

There is evidence that, when viscous solutions of hyaluronate are allowed to react with simple chemicals, such as reducing reagents, hydrolysis stops long before the mono- or disaccharide stage is reached (Hale, 1944). (The results obtained by Skanse & Sundblatt (1943), indicating the formation of disaccharides in this type of degradation, could not be confirmed in these laboratories (Hale, unpublished results).) If the chemical nature of the oligosaccharide resulting from this relatively slight hydrolysis of the molecule were known, it might be of considerable help in the selection of suitable substrates for an examination of the precise degree of complexity of the hyaluronidase preparations. Until such knowledge is available, it is unprofitable to draw dogmatic conclusions from studies in which some particular fraction obtained from the polysaccharide is used as a substrate. The purpose of using the two methods chosen in the present study has merely been to indicate in a qualitative manner the complexity of the bacterial hyaluronidases. Further work on the polysaccharide may shed more light on the understanding of the results, as, for example, the recognition of the amylose and amylopectin fractions of starch has helped in studies of the amylases.

SUMMARY

1. An accurate method has been devised for the assay of bacterial hyaluronidase activity with respect to the liberation of reducing sugars from potassium hyaluronate.

2. The pH optima of streptococcal, staphylococcal and *Cl. welchii* hyaluronidases have been measured using this method. The results obtained for the first two enzymes do not agree with those obtained using a viscosity-reduction technique.

3. The relative potencies of samples of streptococcal and staphylococcal hyaluronidase, measured by the liberation of reducing sugars from hyaluronate, do not agree with measurements made by the viscosity-reducing technique.

4. It is deduced that preparations of streptococcal and staphylococcal hyaluronidase contain several enzymes responsible for various stages in the degradation of hyaluronate.

5. Highly potent hyaluronidase can be produced by growing streptococci inside dialysis sacs surrounded by medium containing no hyaluronate.

I should like to express my thanks to Dr Bergel of Roche Products Ltd., through whose continued courtesy supplies of growth factors have been available, and to Miss P. Fitch for considerable and effective technical assistance given during this stage of the work.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **260th Meeting** *of the Biochemical Society was held at the School of Biochemistry, Tennis Court Road, Cambridge, on Saturday,* 27 *September* 1947, *at* 11 *a.m. when the following papers were read:*

COMMUNICATIONS

Correlation between Penicillin Resistance and the Ability to Assimilate Glutamic Acid in *Staphylococcus aureus*. By E. F. Gale. (*Biochemical Laboratory, University of Cambridge*)

Gram-positive cocci assimilate glutamic acid in the free state and concentrate it in the internal environment prior to metabolism (Gale, 1947). Penicillin impairs the assimilatory process and eventually blocks the passage of glutamic acid across the cell wall (Gale & Taylor, 1946). Organisms which are resistant to penicillin require correspondingly larger amounts of penicillin to block the assimilatory process. The internal concentration of glutamic acid achieved within the cell depends upon the external concentration when this falls below a certain level; the assimilation constant can be defined as that external concentration of glutamic acid which gives rise to an internal concentration half that attained at saturation. The reciprocal of the assimilation constant can be taken as a measure of the affinity of the cell for glutamic acid.

A study of the assimilation constants of a series of organisms of differing penicillin sensitivities shows that the two factors are related in that organisms with high penicillin resistance have large assimilation constants. The correlation was proved by culturing a strain of *Staph. aureus* in increasing concentrations of penicillin and determining its assimilation constant at resistance levels of 15, 60, 200 and 2000 units/ml. As the resistance increases the affinity of the cell for glutamic acid decreases. It follows that the cell would have to change the nature of its metabolism if it were to continue to grow at very high penicillin levels. Such a change has recently been reported by Bellamy & Klimek (1947), and the metabolism of such resistant organisms will be discussed.

It appears that there is a definite relation between the assimilation of glutamic acid by the cell and its sensitivity to penicillin, although it may be that the changes demonstrated with glutamic acid may be symptomatic of a general change in the assimilatory properties of the cell.

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The Assimilation of Amino-acids by Yeast. By E. Shirley Taylor (introduced by E. F. Gale)

Further Observations on the Steroids of Pregnant Mares' Urine. By W. Klyne and J. Y. F. Paterson. (*Department of Biochemistry, University of Edinburgh*)

Compound Z previously isolated from pregnant mares' urine as its sulphate (Klyne & Marrian, 1945; Klyne, 1946) has been identified as *allo*-pregn-16-ene-3(β)-ol-20-one by comparison with authentic material (Plattner, Ruzicka, Heusser & Angliker, 1947).

Compound Y, a saturated diol $C_{21}H_{36}O_2$, which was obtained as its sulphate (Klyne, 1946) and which is probably identical with uranediol (Marker, Rohrmann & Wittle, 1938), has been further studied. Its chromic acid oxidation product 'Y ketone', $C_{21}H_{32}O_2$, ($[\alpha]_D^{20^\circ} = -21^\circ \pm 1^\circ$, chloroform) is not changed by boiling with ethanolic HCl, and on Clemmensen reduction yields a hydrocarbon (m.p. 122–124°, corr.) (cf. urane, m.p. 128°, Marker, Kamm, Oakwood, Wittle & Lawson, 1938).

The conjugates from mares' pregnancy urine which could not be purified as such were hydrolyzed with acid and the hydrolysate fractionated by the usual alkali, Girard and digitonin separations. Part of the non-phenolic fraction was insoluble in benzene.

This material after acetylation and chromatography (Al_2O_3) was fractionally crystallized from methanol and aqueous methanol (Marker, Kamm, Crooks, Oakwood, Wittle & Lawson, 1938) to yield the pregnanetriol triacetate of Haslewood, Marrian & Smith (1934), (m.p. 164–166°, corr., $[\alpha]_D^{23^\circ} = -45^\circ \pm 1^\circ$, chloroform) and another compound, T acetate, which is probably the uranetriol triacetate of Marker, Kamm, Crooks *et al.* (1938):

	m.p.
Cpd. T	296–298° (uncorr.)
Cpd. T acetate	135–136° (corr.)
Cpd. T ketone (CrO_3 oxidation product)	223–226° (corr.)

Chromatography (Al_2O_3) of the non-phenolic, non-ketonic digitonin-precipitable fraction has yielded two new compounds. Compound W appears to be a saturated diol; its acetate has the probable formula $C_{25}H_{40}O_4$, m.p. 175·5–178° (corr.), $[\alpha]_D^{19^\circ} = -54^\circ \pm 1^\circ$ (chloroform). These properties do not agree with those of the acetate of any known C_{21} diol having a 3(β)-hydroxy group. Compound V appears to be a saturated monohydroxy steroid, m.p. 61–63° (corr.).

	m.p.
Uranetriol	295–300°
Uranetriol acetate	136°
Uranetrione	245°

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The Isolation from the Urine of Pregnant Mares of *allo*-Pregnan-3(β)-ol-20-one sulphate.

By J. Y. F. Paterson and W. Klyne. (*Department of Biochemistry, University of Edinburgh*)

The urine was extracted by the method previously described (Schachter & Marrian, 1938; Klyne & Marrian, 1945). The 'water-insoluble', 98 % acetone-soluble, material gave, with piperazine dihydrochloride, the crystalline salt of a sulphate, m.p. 240–241° (dec., corr.), probable formula

$$(C_{21}H_{33}O\,.\,SO_4^-)_2\,.\,(C_4H_{12}N_2^{++}).$$

This sulphate was hydrolyzed with boiling dilute hydrochloric acid, and the product treated with digitonin. The digitonin-precipitable material, after acetylation, was chromatographed (Al_2O_3), yielding X acetate, $C_{23}H_{36}O_3$, m.p. 140–141° (corr.), $[\alpha]_D^{22^\circ} = +72^\circ \pm 1^\circ$ (chloroform). X acetate was saturated to tetranitromethane, and gave a positive Zimmerman ketosteroid reaction. Hydrolysis of the acetate gave a substance X, $C_{21}H_{34}O_2$, m.p. 190–191° (corr.), $[\alpha]_D^{20^\circ} = +90^\circ \pm 1^\circ$ (chloroform).

These melting-points and rotations agree with those of *allo*-pregnan-3(β)-ol-20-one and its acetate (Meystre & Miescher, 1946), which were prepared from the acetate of pregn-5-ene-3(β)-ol-20-one (Plattner, Heusser & Angliker, 1946). Compound X and its acetate on mixing with *allo*-pregnan-3(β)-ol-20-one and its acetate respectively gave no depression of melting-point.

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The Production of Propionic Acid by Decarboxylation of Succinic Acid in a Bacterial Fermentation.

By A. T. Johns (introduced by M. Stephenson). (*Department of Biochemistry and the A.R.C. Unit of Animal Physiology, University of Cambridge*)

In the course of a survey of the organisms responsible for the production of propionic acid in the rumen of the sheep a new micrococcus has been isolated in pure culture. This organism is a strict anaerobe and differs from the *Propionibacteria* in that it ferments no sugar tested, but produces propionic acid along with acetic acid, H_2 and CO_2 from lactic acid. A survey of possible fermentation substrates showed that it attacks pyruvic, oxalacetic, *l*-malic and fumaric acids with the production of propionic, acetic and carbonic acids and H_2, but that the fermentation of succinic acid gives propionic acid and CO_2 as sole products.

Barker & Lipmann (1944) have put forward evidence that lactate does not form an intermediate in the reduction of pyruvic to propionic acid. The appearance of C^{13} in the carboxyl group of propionic acid formed during fermentation by *Propionibacteria* in the presence of $C^{13}O_2$ led Wood, Werkman, Hemingway & Nier (1941) to suggest that propionic acid may arise via the Wood-Werkman CO_2 fixation scheme by the decarboxylation of one of the dicarboxylic acids, possibly succinic. Such a reaction has, however, never been shown as a result of fermentation by true *Propionibacteria*. The findings noted above with this micrococcus would be in general agreement with the scheme postulated above, and consequently attention has been concentrated on the decarboxylation of succinic acid by this organism.

Washed suspensions of cells produce a simple decarboxylation of succinic acid giving 97 % of the theoretical yield of CO_2 according to the equation

$$\mathrm{HOOC{-}CH_2{-}CH_2{-}COOH} \rightarrow \mathrm{CH_3{-}CH_2{-}COOH + CO_2}.$$

Investigation of the nature of the acid product by the chromatographic method (Elsden, 1946) gives 100·1 % yield propionic acid and no other product. The reaction can be studied manometrically at an optimum pH of 7·4 when Q_{CO_2} values of the order 40–50 are obtained at 37°.

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The Decarboxylation of β-(2:5-dihydroxyphenyl)-alanine. By H. Blaschko and G. H. Sloane Stanley. (*Department of Pharmacology, University of Oxford*)

Extracts containing the enzyme dopa decarboxylase (rat's liver and guinea-pig's kidney) will decarboxylate DL-2:5-dihydroxyphenylalanine. The rate of the reaction is slightly slower than with the 3:4-acid; the total CO_2 formation suggests that only one stereoisomeride is decarboxylated.

This observation is of interest in connexion with tyrosine breakdown. Neuberger, Rimington & Wilson (1947) have shown that in human alcaptonuria the 2:5-acid is converted to homogentisic acid; their quantitative data suggest that the L-form of the acid is an intermediate in homogentisic acid formation. The possibility of a pathway, tyrosine → 2:5-dihydroxyphenylalanine → 2:5-dihydroxyphenylethylamine → homogentisic acid, has therefore to be considered (see Neubauer, 1928); our results show that a mammalian enzyme exists which will catalyse one of these steps.

The acid is not markedly oxidized by the ophio-oxidase of cobra venom.

The authors are grateful to Dr A. Neuberger for the 2:5-dihydroxyphenylalanine used in these experiments.

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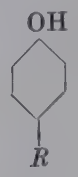
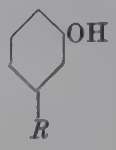
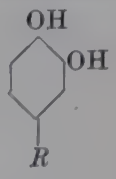
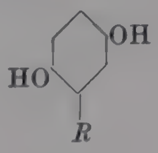
Decarboxylation of Tyrosine and its Derivatives by Mammalian and Bacterial Enzymes. By H. Blaschko and G. H. Sloane Stanley. (*Department of Pharmacology, University of Oxford*)

Tyrosine is not decarboxylated by the mammalian dopa decarboxylase, but Epps (1944) has shown that the tyrosine decarboxylase from *Streptococcus faecalis* will also act on 3:4-dihydroxyphenylalanine. An analysis of this difference shows that the mammalian enzyme requires the presence of a phenolic hydroxyl group in the *meta* position, the bacterial enzyme one in the *para* position. Both *m*-tyrosine and 2:5-dihydroxyphenylalanine are decarboxylated by extracts containing dopa decarboxylase, but not by the bacterial enzyme.

meta-Tyrosine is a substrate of ophio-oxidase of cobra venom and of D-amino-acid oxidase of pig's kidney.

The authors are grateful to Prof. I. C. Gunsalus for the strain of *Str. faecalis* (A.T.C.C. no. 4083) and to Dr H. R. Ing for the *meta*-tyrosine used in these experiments.

Substrate:	Tyrosine	*meta*-Tyrosine	3:4-Dihydroxy-phenylalanine	2:5-Dihydroxy-phenylalanine
				
Guinea-pig's kidney	−	+	+	+
Streptococcus faecalis	+	−	+	−

$R = \text{—}CH_2.CH(NH_2)COOH.$

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Increased β-Glucuronidase in Response to Tissue Injury.

By G. A. Levvy, Lynda M. H. Kerr and J. G. Campbell (introduced by G. F. Marrian). (*Department of Biochemistry, University of Edinburgh and Royal (Dick) Veterinary College, Edinburgh*)

Fishman (1940) found that repeated feeding of menthol to mice caused statistically significant increases in β-glucuronidase in liver, spleen and kidney. He postulated this enzyme to be responsible for glucuronide synthesis *in vivo*, and suggested that he was measuring adaptation by the enzyme in response to the presence of excess substrate. Menthol is known to be excreted as the glucuronide in certain species, but it has not been established that β-glucuronidase acts synthetically.

Using a new method for assay of the enzyme (Kerr, Graham & Levvy, 1947), an attempt was made to confirm Fishman's findings. Twenty-four hours after intraperitoneal injection of *l*-menthol in oil into mice, there was a marked rise in glucuronidase in liver, but not in kidney and spleen. Gross liver damage was noted, and it was considered that this might explain the rise in the enzyme. On histological examination, fatty degeneration, necrosis and evidence of repair were seen in the liver, while the kidneys and spleen showed little deviation from normal.

A variety of substances known to produce liver or kidney damage, but in most cases unlikely to form glucuronides, were injected into mice and liver, kidney and spleen were examined histologically and for their glucuronidase content. Carbon tetrachloride and *l*-menthol-β-D-glucuronide resembled menthol in their effects. Chloroform behaved in the same way as menthol with regard to liver and spleen, but produced severe damage in kidney unaccompanied by any rise in the enzyme. Mercuric nitrate produced profound damage in kidney, followed by rapid repair and a rise in glucuronidase. It had little effect in either respect on liver and spleen. Phenylarsenoxide and yellow phosphorus caused severe liver damage with little evidence of repair. The former had no effect on the glucuronidase level in liver, and the latter depressed it, favouring the view that a rise in the enzyme is associated with repair rather than damage. The results obtained for kidney after injecting chloroform could be explained likewise.

A further series of experiments was carried out with relatively non-toxic compounds known or considered to give rise to glucuronides *in vivo*. Neither pregnanediol, sulphathiazole nor pregnanediol-β-D-glucuronide affected the glucuronidase level in liver, spleen or kidney. No pathological changes in these organs were observed after injection of the first two compounds.

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Observations on the Decarboxylation of Glutamine by *Clostridium welchii*. By H. A. Krebs

Clostridium welchii, strain S.R. 12, specifically decarboxylates glutamic acid (Gale, 1945) and glutamine (Gale, 1947). Glutamine reacts much more slowly than glutamic acid, especially at low concentrations of the substrate. At pH 4·5, for example, glutamic acid reacted quantitatively in 15 min., whilst glutamine required 80 min. At pH 4·0 the differences were even greater. The rate of reaction of glutamine is much accelerated by the detergent cetyltrimethyl ammonium bromide ('cetavlon'), 0·02 %. At pH 4·0 the acceleration was about 20-fold. Plasma protein, or protein containing tissue extracts, also accelerated the decarboxylation of glutamine, though much less than cetavlon. The decarboxylation of glutamic acid was also accelerated by cetavlon, but owing to the relatively high rate of the decarboxylation without detergent the percentage increase was in this case smaller than with glutamine. An anionic detergent, sodium dodecylsulphate, inhibited the decarboxylation of glutamine and glutamic acid. In another strain of *Cl. welchii* which contained histidine and glutamic decarboxylases cetavlon likewise accelerated the glutamic decarboxylase but inhibited the histidine decarboxylase, and sodium dodecylsulphate had no effect on the histidine decarboxylase.

The decarboxylation of glutamine is preceded by the conversion into ammonium glutamate. The glutaminase of strain S.R. 12 is highly specific. No other substance has so far been found to yield ammonia when incubated with a washed suspension of the organism at pH 4·9. This glutaminase preparation is more convenient to make than Archibald's (1944) glutaminase preparation from kidney and is more specific. It can be used for the determination of glutamine on Archibald's principle.

An acceleration of the decarboxylation of glutamic acid and glutamine by cetavlon was also found in *Escherichia coli* and *Proteus morganii*.

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Paper Chromatography of Flavin Nucleotides. By J. L. Crammer

Adenosinetriphosphate in Glutamine Synthesis. By W. H. Elliott (introduced by M. Dixon). (*Biochemical Laboratory, University of Cambridge*)

It was shown by Krebs (1935) that the energy required for the synthesis of glutamine from glutamic acid and NH_3, which occurs in tissue slices of brain cortex, can be furnished by the oxidation of glucose. During the course of a recent survey for phosphate transferring enzymes, evidence has been obtained that adenosinetriphosphate (ATP) is the immediate energy donor for glutamine synthesis. Since this work was performed, Speck (1947), in a recent publication, has reported similar results obtained with pigeon liver.

An enzyme which synthesizes glutamine has been extracted from acetone-dried grey matter of sheep's brain. The system requires the presence of Na (L)-glutamate, NH_3 and ATP. When hydroxylamine is added instead of NH_3, the corresponding hydroxamic acid is formed, which can be conveniently estimated colorimetrically by the method of Lipmann & Tuttle (1945). Using an enzyme preparation freed from adenosinetriphosphatase activity, it was shown that the formation of hydroxamic acid is accompanied by the liberation of an equivalent amount of inorganic phosphate from ATP. A similar liberation of phosphate occurs in the presence of NH_3 during the formation of glutamine.

It seems possible that the first stage of glutamine synthesis might be a transfer of phosphate from ATP to the γ-COOH of glutamic acid forming glutamyl-phosphate. This could then react with either NH_3 or hydroxylamine to form glutamine or hydroxamic acid respectively. The enzyme resembles known phosphokinases in requiring magnesium ions and cysteine for its activity. It is completely specific for the natural isomer of glutamic acid.

The enzyme has also been found in a cell-free extract obtained from *Staphylococcus aureus*. The bacterial enzyme preparation is completely inhibited by crystal violet in a concentration of 1 in 10,000 (0·00025M). This can be correlated with the results obtained by Gale & Mitchell (1947), who showed that crystal violet and other dyes of the triphenylmethane series suppress some phase of glutamic acid metabolism. Methionine-sulphoxide also inhibits the enzyme.

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The Amino-acid Composition of Salmine. By G. R. Tristram. (*School of Biochemistry, University of Cambridge*)

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Persistence of Suramin Analogues in the Blood Stream. By A. Spinks. (*I.C.I. Ltd., Blackley, Manchester*)

In the hope of correlating the remarkable persistence of suramin with its molecular structure, eleven related compounds have been studied. They include the three constituent amino-acids of suramin, and peptide-like compounds and ureas of varying complexity prepared from these. Methods of determining each have been devised, and each has been administered to rabbits intravenously in doses of 100 mg./kg. The resulting blood concentration-time curves show two main trends. First, persistence tends to increase with increasing molecular complexity; and, secondly, compounds containing naphthylaminepolysulphonic acids as end-groups tend to show higher persistence than analogous carboxylic acids. For persistence of the suramin order the presence in the molecule of two naphthylaminepolysulphonic acid groups, and a total number of six amino-acid residues appears to be necessary. *m*-Aminobenzoic, *m*-amino-*p*-toluic, and sulphanilic acid groups may be interchanged within this basic design without marked effect on the persistence.

Since the compounds examined include most of the possible hydrolysis products of suramin, and since these were rapidly eliminated it is probable that suramin is not markedly hydrolyzed *in vivo*. No relation between persistence and activity was found. It is probable that these two properties are conferred by different structural features of the molecule.

The Estimation of Suramin (Antrypol, Bayer 205) in Plasma. By J. C. Gage, F. L. Rose and Mary M. Scott. (*I.C.I. Ltd., Blackley, Manchester*)

A colorimetric method of estimating suramin has been developed, based on an observation that the red colour of certain styryl-quinoline dyes is discharged by the drug. Plasma proteins do not interfere with the reaction; the estimation of suramin in plasma is simpler and more rapid than the hydrolysis method of Dangerfield, Gaunt & Wormall (1938) and attains a higher sensitivity. The plasma concentration curve obtained by this method from a rabbit dosed intravenously with the drug is similar to that obtained by the hydrolysis method. A number of the possible hydrolytic fission products of suramin have been examined and positive results by the method have been found with those containing the naphthalene trisulphonic acid residue.

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Changes in Nicotinic Acid Content of Cereals occurring during Germination.

By CHLOE KLATZKIN, F. W. NORRIS and F. WOKES. (*Ovaltine Research Laboratories and the Department of Industrial Fermentation, University of Birmingham*)

James, Norris & Wokes (1947) have recently described a chemical method of estimating nicotinic acid which gives fairly good agreement with microbiological assays on a variety of foods. This chemical method has now been used to estimate nicotinic acid in different cereals before and after germination, in parallel with microbiological assays.

Barley, wheat, maize and oats have been malted by the large-scale procedure normally used for barley in this country, and the nicotinic acid content determined by chemical and microbiological methods at intervals during the process of malting. All these cereals, and rice, have also been germinated in the laboratory, at room temperature and at 37°, and their nicotinic acid content estimated. Changes in the average dry weight per seed have been determined, and correction for these changes applied when calculating the nicotinic acid content per seed.

Using both chemical and microbiological assays, no increases in the nicotinic acid content have been observed during the germination of barley or maize and only slight increases during the malting of wheat. This agrees with the findings of Davis, Laufer & Saletan (1943), who used microbiological methods only. Appreciable increases have been found to occur during the germination of oats and rice.

The very large increases found to occur in oats by American workers (Burkholder, 1943; McVeigh, 1944), using microbiological methods of assay only, have not been confirmed. These large increases may be due to their having obtained a more rapid growth of seedlings, since the average loss in dry weight per seed during 5 days in their experiments is much greater than the corresponding loss during 12 days in our experiments.

However, experiments on oats, barley and maize germinated at 37° in our laboratory failed to show any greater increase in nicotinic acid content per seed than we had obtained under normal malting conditions. This was in spite of our obtaining a greater degree of germination, as shown by weight changes and increases in length of the shoot (acrospire).

Emphasis is laid on the importance of noting the degree of germination, by measuring acrospire ratios (A.O.A.C. 1945). With rice in particular, seeds which had germinated further during 9 days had a considerably higher nicotinic acid content per seed than those which had germinated less in the same period.

A marked discrepancy has been found between chemical and microbiological results when assaying nicotinic acid in oats. Further work is being carried out on this problem. It is concluded that some precursor of nicotinic acid is present in wheat, rice and oats.

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The Fate of 2:4-Dimethyl-3-carbethoxy-pyrrole-5-carboxylic Acid and 2:4-Dimethyl-5-carbethoxy-pyrrole-3-carboxylic Acid in the Rabbit.

By C. H. GRAY and E. F. J. THORPE. (*Biochemical Department, King's College Hospital, S.E. 5*)

2:4-Dimethyl-3-carbethoxy-pyrrole-5-carboxylic acid in the form of its sodium salt was dissolved in water and administered by stomach tube to adult male rabbits fed on a stock diet. The urine subsequently collected was found to give a positive reaction for pyrroles and to reduce Benedict's solution. A glucuronide was isolated by extraction of the urine with methyl ethyl ketone after saturating with sodium chloride and acidification with hydrochloric acid. The ketone layer was washed with a little water and taken to small volume *in vacuo* when fractional precipitation with ether gave a reasonably pure specimen of the glucuronide. Recrystallization from hot water yielded the glucuronide m.p. 163°, equivalent weight 382 (theoretical 387). Diazomethane gave a methyl ester m.p. 171°. The glucuronide was soluble in the lower aliphatic alcohols and acetone but insoluble in most other organic solvents with the exception of hot ether, in which it was slightly soluble and from which it could be crystallized in the form of needles. Benedict's solution was readily reduced. The glucuronide became rapidly discoloured on contact with the atmosphere, particularly in aqueous solution.

The isomeric 2:4-dimethyl-5-carbethoxy-pyrrole-3-carboxylic acid was likewise fed to rabbits, and it was found possible by a similar technique to isolate the corresponding glucuronide. The 3-glucuronide (m.p. 146°, methyl ester 184°) was more soluble in water and not quite as stable as the 5-isomer.

Molecule for molecule the colour produced by the glucuronide with Ehrlich's *p*-dimethyl-aminobenzaldehyde reagent was equivalent to that produced by the original acid. The total pyrroles present in the urine were therefore determined using the colour developed with Ehrlich's reagent, and the increase in excretion of glucuronic acid also measured by the method of Hanson, Mills & Williams (1944).

About 50% of the dose fed was excreted as the glucuronide in the case of 2:4-dimethyl-3-carbethoxy-pyrrole-5-carboxylic acid and about 30 % in the case of 2:4-dimethyl-5-carbethoxy-pyrrole-3-carboxylic acid; in the latter case about 20% of the dose could be recovered unchanged. The fate of the remainder of the administered material is at present unknown, but it is probably metabolized.

		Amount of glucuronide recovered	
Exp.	Dose (g.)	Using Ehrlich's reagent (g.)	By naphtho-resorcinol method (g.)
2:4-Dimethyl-3-carbethoxy-pyrrole-5-carboxylic acid			
1	1·6	1·5	1·2
2	1·0	0·84	0·97
3	1·3	0·55	0·7
2:4-Dimethyl-5-carbethoxy-pyrrole-3-carboxylic acid			
1	1·6	0·98	0·72
2	1·6	0·8	1·14
3	1·6	1·08	1·28

Only traces of pyrrolic material could be detected in the faeces. No urobilinoid or bilirubinoid substances could be detected in the urine using Schlesinger's and Fouchet's tests.

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The Preparation of Coproporphyrin III from Toxic Filtrates of *Corynebacterium diphtheriae* Cultures. By C. H. Gray and L. B. Holt. (*Biochemical Department, King's College Hospital, S.E. 5 and the Wright-Fleming Institute for Microbiology, W. 2*)

Corynebacterium diphtheriae was grown on a slightly modified Mueller's hydrolyzed casein medium, and after 7 days' growth the organisms were filtered off. The toxic supernatant fluid was treated with a magnesium hydroxide gel, and after standing the resulting precipitate, which contained all the porphyrin, was separated. The supernatant possessed an almost undiminished *Lf* value and was subsequently used for the preparation of toxoid by treatment with formaldehyde. The red magnesium hydroxide precipitate was then treated with 10N-hydrochloric acid, and the porphyrin solution thus obtained was separated from the protein precipitate by centrifuging. A second hydrochloric acid extract of the protein precipitate was added to the main porphyrin solution, which was then adjusted to pH 3·3 by the addition of sodium hydroxide solution. The porphyrin precipitated quantitatively and was centrifuged off. After two repetitions of this precipitation at pH 3·3 from hydrochloric acid solution, the porphyrin was centrifuged off, dried *in vacuo* and esterified with methyl alcohol saturated with hydrochloric acid gas. After transferring the ester to chloroform in the usual way, the chloroform solution was washed with water, dilute ammonia and again with water and then evaporated to dryness to give the crude ester.

The crude ester was dissolved in benzene and the solution passed through a column of calcium carbonate. The bulk of the porphyrin ester passed slowly through the column as an entity and proved to be pure coproporphyrin III tetramethyl ester with the properties previously described (Gray & Holt, 1947). A second zone left on the column required for elution benzene containing 20% (v/v) chloroform, and proved to be uroporphyrin I octamethyl ester, m.p. 288°. A third zone required pure chloroform for its elution, and although possessing the properties of a porphyrin ester could not be identified. A fourth brown zone left on the column was not a porphyrin.

From 130 l. of *C. diphtheriae* filtrate, 479 mg. of pure coproporphyrin III ester and 7 mg. of pure uroporphyrin I ester were isolated. Since these materials are readily obtainable without significant loss of toxin, the preparation of coproporphyrin III ester in large quantities in the large-scale production of diphtheria toxoid provides a new and hitherto unexplored source of coproporphyrin III. In the large-scale preparation of purified diphtheria toxoid, it is more convenient to perform the magnesium hydroxide precipitation after conversion of the toxin to toxoid.

REFERENCE

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The Formation of Nicotinamide by *B. coli*. By P. Ellinger and M. M. Abdel Kader. (*Lister Institute, London, S.W.* 1)

The synthesis of nicotinamide or nicotinic acid by a number of bacteria in pure culture has been shown by Burkholder & McVeigh (1942) and Thompson (1942). Recently Ellinger & Emmanuelowa (1946) have shown that intestinal biosynthesis of nicotinamide in human beings is mainly due to *B. coli*, and Ellinger, Abdel Kader & Emmanuelowa (1947) have examined *in vitro* this synthesis by *B. coli*.

To study the mechanism of this biosynthesis various strains of *B. coli* were incubated in ammonium lactate (Fildes, 1938), and the nicotinamide formed was measured microbiologically (Barton-Wright, 1944). Then one of the following amino-acids was added before incubation: L(+)-ornithine, L(+)-arginine, D, L-glutamine, D, L-glutamic acid, L(+)-citrulline, L(+)-proline, L(+)-leucine, L(−)-isoleucine, D, L-valine or D, L-tryptophane. The examination for nicotinamide formed revealed that concentrations of 10^{-3} to 6×10^{-4}M of ornithine increased the nicotinamide formation up to $4\frac{1}{2}$ times, while glutamine and arginine gave an increase of 50–70% when compared with the synthesis of nicotinamide in the control. All the other amino-acids either did not affect the nicotinamide formation or inhibited it. With the help of a strain of *Leuconostoc mesentroides*, kindly supplied by Dr B. C. Johnson of the University of Illinois, which was less sensitive to nicotinamide than nicotinic acid, we could establish that almost all the synthesized material was nicotinamide and not nicotinic acid. Preliminary experiments with washed and disintegrated *B. coli* showed that in saline solution some nicotinamide was formed from ornithine. This formation was inhibited by NaCN (10^{-4}M). The experiments confirm the suggestion of Guggenheim (1940) that ornithine might be the basis of the biosynthesis of nicotinic acid.

Whether this mechanism is involved in the formation of nicotinamide from tryptophane and whether ornithine is formed from tryptophane needs further investigation. Tryptophane did not increase the formation of nicotinamide by *B. coli*. This might be due to the fact that *B. coli* are unable to disintegrate tryptophane to ornithine. The results of preliminary experiments carried out with a mixed culture of intestinal bacteria from the rats' caecum support this hypothesis. If an emulsion of this culture is incubated in Barton-Wright medium with tryptophane an increased biosynthesis of nicotinamide is observed.

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The Biosynthesis of a Member of the Folic Acid Group of Factors. By D. A. Hall. (*School of Medicine, Leeds* 2)

Enzyme systems capable of deaminating histidine are reported by Edlbacher & Neber (1934) as present in liver, and by Borghi & Tarrantino (1938) in skin tissue. Members of the folic acid group of factors have long been known to be present in liver tissue, and the present author has also shown that skin tissue contains folic acid-active material. In view of this correlation and the observations previously reported (Hall, 1947) regarding the production of a member of the folic acid group by chemical means from histidine, it was considered desirable to determine whether certain components of the enzyme systems mentioned above were capable of producing activity from histidine.

It was shown that an aqueous extract of mouse skin, when incubated at pH 6·74 at 37° with histidine, was capable of stimulating the growth of cultures of *S. Lactis* R at lower concentrations than when treated under similar conditions in the absence of histidine. A tenfold increase in activity was obtained after 5 hr. incubation. Similar results were obtained with aqueous or M/20-phosphate buffer (pH 6·74) extracts of both mouse and cat liver, and the effect was shown to be truly enzymatic, and not due to liberation of bound activity, by the inactivation of the preparations by boiling. Attempts to separate the responsible components from the total liver extract are not complete, but it has been shown that the activity is not connected with that fraction soluble in half-saturated ammonium sulphate solution containing vitamin B_c conjugase. This fraction is, however, required as well as the precipitate for the reaction to proceed. Edlbacher & Neber (1934) and Walker & Schmidt (1944) postulated the production of unstable intermediates of the type-formamidino glutaric acid or formyl glutamine, and it may be possible that other components of the liver extract are capable of catalyzing the re-combination of small quantities of these intermediates with the production of pterin-like nuclei. Arginine, which is of similar structure to these intermediates, will also act as a substrate for the production of folic acid like activity, but glutamic acid, the end-point of the degradation of histidine, is not equally effective.

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DEMONSTRATIONS

Some Characteristics of Penicillin-resistant Staphylococci. By E. F. Gale

A New Micro-electrophoresis Cell. By P. D. Mitchell (introduced by E. Baldwin)

The **261st Meeting** *of the Biochemical Society was held at St Thomas's Hospital Medical School, London, S.E.* 1, *on Friday*, 31 *October* 1947, *at* 2.15 *p.m., when the following papers were read:*

COMMUNICATIONS

An Apparatus for the Preparative Electrophoretic Separation of Amino-acids, Peptides and Proteins. By J. A. V. Butler and Joan M. L. Stephen. (*Courtauld Institute of Biochemistry, The Middlesex Hospital, W.* 1)

Nearly all of the considerable number of arrangements which have been proposed for separating substances having different electric mobilities have serious disadvantages of one kind or another. Diaphragm cells as used by Gordon, Martin & Synge (1941), among others, with three or more compartments, are useful for rough separations of substances moving in opposite directions, but are useless for taking advantage of differences of mobility in the same direction since convective mixing occurs in the cells and the useful separative process is confined to the membranes. The Tiselius apparatus is essentially one for the analysis of the moving fronts, and though samples can be withdrawn for analysis it does not permit the complete separation of the components of a mixture (Theorell, 1934; Svensson, 1946; Bunn, 1947). Various methods of stopping convection on the electrolyte column have been suggested, e.g.

silica gel (Consden, Gordon & Martin, 1946), which has been effectively used for separation of amino-acids and the lower peptides; the use of tubes packed with sintered glass (Coolidge, 1939), which however, confers a very high resistance on the column.

We have developed an apparatus in which migration occurs down a tube, which is packed with a loose filling material, such as asbestos fibre of the type used in Gooch crucibles, glass wool, or cotton fibres. These do not greatly increase the resistance of the column of electrolyte and effectively prevent convection. The filter can be divided into sections by filter-paper barriers at 1 in. intervals, which permit the wads to be extracted from the tube and easily separated from each other. The liquid contents of each segment can then easily be squeezed into a test-tube for analysis.

The tubes (of a polystyrene plastic material) are attached to completely closed silver chloride electrodes of the type used with the Tiselius apparatus so that there is no electro-endosmotic flow through the apparatus. Good separations have been obtained with mixtures of amino-acids, of amino-acids and peptides and of two proteins. In the latter case glass wool or cotton had to be used as the proteins were adsorbed on asbestos.

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Synthesis of Phospholipids in the Foetus.

By G. Popják, Beit Memorial Fellow for Medical Research. (*Department of Pathology, St Thomas's Hospital Medical School, London, S.E.* 1)

Two to six hours after pregnant rats, rabbits and guinea-pigs were injected with Na_2HPO_4 labelled with P^{32}, the specific activity of phospholipid-P extracted from foetal livers and foetal placentae of all three species was much greater than that of the maternal plasma phospholipid-P. If foetal phospholipids were derived from the preformed maternal substances by placental transmission only, the specific activity of foetal phospholipids should not exceed the activity of the maternal plasma lipid. The findings, therefore, indicate that the foetal liver and foetal placenta synthesize phospholipids. When the mothers were injected with the labelled PO_4 the radioactivity of the phospholipids extracted from foetal tissues other than liver and placenta was less than that of the maternal plasma lipid. The lower specific activity of these foetal phospholipids was due to the fact that only very small amounts of labelled inorganic PO_4 had accumulated in these tissues. In a few experiments the foetuses were injected with labelled Na_2HPO_4 directly *in utero*. In these experiments no radioactivity could be detected in the maternal plasma phospholipids, yet all foetal tissues examined contained labelled lipids. Preliminary measurements indicate that the turn-over rate of phospholipids in the foetal liver and placenta is higher than in the maternal liver; in other foetal tissues, however, it is approximately the same as in the maternal liver, which is the most active organ in the adult animal in this respect.

Separation of Acidic Amino-acids by Means of a Synthetic Anion Exchange Resin.

By R. Consden, A. H. Gordon and A. J. P. Martin. (*Wool Industries Research Association, Leeds* 6)

The synthetic anion exchange resin, Amberlite IR-4 was used by Cannan (1944) to separate aspartic and glutamic acids together from the other amino-acids in a protein hydrolyzate. By using the resin chromatographically at a suitable pH, the method has been extended to separate aspartic from glutamic acid. Where these amino-acids are present in a protein hydrolyzate, a preliminary separation from the basic and neutral amino-acids is necessary. This is achieved on a chromatogram of the resin maintained at pH 3–4, which preferentially retains the dicarboxylic amino-acids. At this low pH, the resin is sufficiently charged to compete successfully with the basic amino-acids for the acidic amino-acids. After elution with N-HCl, the dicarboxylic acids (up to 5 mg./g. resin) are transferred to a chromatogram at pH 2·5. At this pH, the glutamic acid is first eluted, followed by aspartic acid. The progress of elution in both stages is followed by partition chromatography on paper (Consden, Gordon & Martin, 1944) of small samples of the eluates. Recoveries of 91 and 96 % respectively, of glutamic and aspartic acids were obtained from a synthetic mixture simulating a wool hydrolyzate, using the copper phosphate titration method of Pope & Stevens (1939). If cysteic acid is present, this may be separated after elution of the dicarboxylic amino-acids at pH 2·5 by elution with N-HCl. Alternatively, cysteic acid, because of its much higher net charge at low pH, may be separated in one stage, employing a chromatogram at pH 1·5. In this way, and working on a sufficiently large scale, cysteic acid has been isolated from hydrolyzates of chlorinated wool and was found to be optically active.

These separations take place because of the difference in net charge between the acidic amino-acids at a given pH. In addition to the above factors, successful separations depend on (*a*) the maintenance of a uniform pH in that part of the column in which separations take place, and (*b*) the rate of flow through the columns being kept sufficiently low to ensure equilibration. This is aided by the use of finely ground resin.

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The Site of Formation of Gastric HCl as Determined by Indicators.

By Norah M. Bradford and R. E. Davies. (*M.R.C. Unit for Research in Cell Metabolism, The University, Sheffield* 10)

Claude Bernard (1858) first used microchemical methods in an attempt to locate the site of formation of the HCl produced by gastric mucosa, and Babkin (1944) has reviewed the large amount of work on this topic. There is no convincing evidence for the presence of acid in fresh excised tissue and, although it is generally held that the oxyntic cells secrete HCl at concentrations isotonic with the blood (see Hollander, 1943), some workers (see Hoerr & Bensley, 1936) have stated that a precursor of the acid occurs within the gastric tubules and is hydrolyzed to form HCl in the foveolae.

Frog gastric mucosa can secrete some dyestuffs *in vivo* (Henning, 1932) and HCl *in vitro* (Delrue, 1930). Tied bags of isolated frog gastric mucosa (Davies, 1946) were used to find out which dyestuffs were secreted without interfering with respiration or acid secretion. All of the following basic (chromogen in cation) dyes, of widely differing chemical structure, were transported into the lumen of the mucosal bag from nutrient saline solutions: acridine, acriflavine, azur I, azur II, benzene azo-α-naphthylamine, Bismarck brown, brilliant cresyl blue, brilliant green, dimethyl yellow, malachite green, methylene blue, neutral red, rhodamine B, rhodamine 6G, safranine O, thionine, toluene azo-amino-toluene-2:1:1:4:3 and toluidine blue.

Some of these dyes are acid-base indicators, and microscopic observations were made of small pieces of isolated frog and polecat mucosa which were secreting dye and acid. With neutral red it was found that the lower regions of the mucosa and the cytoplasm of the oxyntic cells were slightly alkaline (pH 7·2–7·6), whilst the canaliculi and the lumen of some of the tubules and the secretory surface of the mucosa were acid (pH $<6·8$). The green fluorescence of acridine in ultra-violet light showed that the contents of some tubules and canaliculi had pH values of $<4·85$, whilst the red colour found when toluene-azo-amino-toluene-2:1:1:4:3 was used indicated a pH $<1·4$ (Kolthoff, 1932). In no case were all the gastric glands found to be actively secreting acid. The site of formation of the acid appeared to be at or near the wall of the canaliculi of the oxyntic cells in the isolated mucosa of both frogs and polecats.

The acid (chromogen in anion) dyes, congo red and phenolphthalein, were not transported, but bromthymol blue, cresol red, fluorescein and methyl orange were sometimes found in traces inside the mucosal bags, especially if their secretions were alkaline or only slightly acid.

Since phenolphthalein is not secreted and is virtually unionized, whilst acriflavine is secreted and is virtually completely ionized at pH 7·4 and below, the theory of Visscher (1942), that dyes pass through cell membranes in undissociated form, does not appear to hold in this case.

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Secretion of Water by Isolated Gastric Mucosa.

By R. E. Davies and C. Terner. (*M.R.C. Unit for Research in Cell Metabolism, The University, Sheffield* 10)

An apparatus has been made and used to study the rates of fluid secretion and of respiration of the isolated gastric mucosa of frogs and toads at 25°. It consists essentially of a constant pressure manometer (modified from Dixon, 1943) and a modified Warburg cup, provided with a hollow stopper to which a graduated capillary side-tube is attached. The manometer is designed to maintain the pressure acting on the nutrient surface of a sheet of mucosa (Davies, 1946) (tied secretory side upwards to the open end of the stopper) at any desired value and to measure the rate of respiration. The volume of liquid secreted is determined from the movements of liquid paraffin in the side-tube.

No secretion of water or acid was observed anaerobically, but small amounts of a neutral liquid traversed the mucous membrane in the direction of applied hydrostatic pressures. Under aerobic conditions both secreting and non-secreting mucosae were largely independent of applied pressures (of the order of ± 50 mm. H_2O). One mucosa produced even 18 cu.mm. secretion/mg. dry wt./hr. against a resultant pressure of 120 mm. H_2O. Neutral red, placed in the nutrient solution, was transported across the mucosa against external pressures.

The addition of thiocyanate (0·01–0·02 M) did not affect the increase of the O_2 uptake of the mucosa which followed stimulation by 5×10^{-5} M-histamine, but strongly inhibited the rate of acid production. The secretion of H_2O was also inhibited; H_2O often 'leaked' a little in the direction of the external pressure for up to 1 hr. before the mucosa resumed secretion at lower but steady rates.

Our results disprove the statement made by Davenport & Fisher (1940) that the energy required for the movement of H_2O 'is doubtless derived from the difference in hydrostatic pressure between the blood and the gastric juice', and are evidence that the transport of H_2O by gastric mucosa is an active secretory process.

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Absorption of Ammonia from the Rumen of the Sheep.

By I. W. McDonald (introduced by E. Baldwin). (*Biochemical Laboratory, University of Cambridge*)

Ammonia in the ruminal contents is derived from the deamination of food proteins and from the conversion of urea in the saliva; a series of analyses showed approximately 10 mg. urea-N/100 ml. saliva. Special interest attaches to the fate of urea in the rumen since urea has been exploited as a source of nitrogen in the nutrition of ruminants; this subject has been reviewed by Smith (1945).

Ammonia could disappear from the ruminal contents (*a*) by utilization by bacteria for their growth, (*b*) by passage in the ruminal ingesta from rumen to abomasum or (*c*) by absorption. Since ammonia-N absorbed from the rumen is probably valueless to the animal for its metabolism, the possibility of absorption assumes some importance.

In experiments on six sheep, it was found that venous blood draining the rumen contained on an average 1·7 mg. ammonia-N/100 ml., while peripheral blood from the jugular vein or carotid artery contained only traces of ammonia. It was further shown that removal of the contents from the rumen resulted in reduction of the ammonia content of the blood draining the organ, while addition of a solution of ammonium acetate to the emptied organ resulted in the reappearance of ammonia in the blood.

Since urea is rapidly converted into ammonia in the rumen (Lenkeit & Becker, 1938), it is now evident that a nitrogen cycle is established in the normal digestive process of the ruminant, viz. urea in saliva → ammonia in ruminal ingesta → ammonia absorbed into portal blood stream → converted into urea by the liver → urea in the general circulation → urea in saliva. Quantitatively it appears that, in the sheep, the amount of ammonia-N absorbed from the rumen will appreciably exceed that of urea-N secreted in the saliva.

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Separation and Estimation of Saturated, Steam-volatile Fatty Acids by Buffered Partition Columns.

By R. SCARISBRICK, E. BALDWIN and V. MOYLE. (*Biochemical Laboratory, University of Cambridge*)

The chromatographic method introduced by Elsden (1946) permits the titrimetric estimation of butyric, propionic and acetic acids (the latter by difference) after their separation from mixtures by means of columns of silica gel impregnated with bromcresol green. A new partition method has now been devised in which heavily buffered silica columns are used. No indicator is employed on the columns, and the analysis is made by titration with N/200-KOH of serial samples of chloroform-butanol elutriates. Naturally occurring fatty acids of the saturated series from acetic to caprylic (C_8) can be separated and estimated in this way, except that no separation is possible between those isomeric forms of butyric and higher acids that have been tested. Caprylic acid is not separable from higher acids, but the bulk of the latter are eliminated by a preliminary steam distillation. Three columns, buffered to different pH's, are required to cover the useful range of the method. The new procedure has considerable advantages over its predecessor; the performance of the columns is not influenced by minor variations in the properties of different samples of silica, the range is substantially greater and the method has considerably greater flexibility. Each acid shows strongly characteristic behaviour on the columns, so much so, indeed, that behaviour may be taken as evidence of identity, except that isomeric pairs, e.g. *n*- and *iso*-valeric acids, cannot be distinguished in this way.

The optimal working load is about 0·1 mmol. of each acid, but the method is still sensitive with loads of only 0·01 mmol. or even less. Loads up to 1 mmol. can be handled, though the separations between adjacent acids are then incomplete. Under optimal conditions 100 ± 4 % of the acids are recoverable, whether put on the columns singly or in the form of mixtures.

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Nucleic Acids Labelled with P^{32} and N^{15}.

By J. N. DAVIDSON and W. RAYMOND. (*Department of Biochemistry, St Thomas's Hospital Medical School, London, S.E.* 1)

Sodium phosphate containing P^{32} was administered to rats in doses of about 10 μc. per animal and the livers were removed 2–4 hr. later. Cytoplasmic and nuclear material were separated and the nucleic acids isolated from each fraction. The phosphorus of the ribonucleic acid was more active than that in the deoxyribonucleic acid, the ratio of the specific activities being about 7 : 1. Similar results were obtained with rabbits. Brues, Tracy & Cohn (1944) obtained figures of this order, while even higher ratios were found by Hammarsten & Hevesy (1946).

In regenerating rat livers the activity of both types of nucleic acid was increased but especially that of the deoxyribonucleic acid, so that the ratio of the specific activities fell to between 2 and 3. In foetal liver the specific activity of the deoxyribonucleic acid frequently exceeded that of the ribonucleic acid.

When ammonium citrate containing 4·18 atom % excess N^{15} was fed to pigeons and rats in doses of 50 mg./day for 3 days, the ribonucleic acid isolated from the pigeon liver contained 0·048 atom % excess N^{15} and the deoxyribonucleic acid only 0·003 atom % excess. The figure for the uric acid excreted in the last 24 hr. was 0·204 atom % excess N^{15}. The ribonucleic acid isolated from rat liver contained 0·053 atom % excess N^{15}, while the amount of excess N^{15} in the deoxyribonucleic acid was negligible.

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The Nucleic Acid Content of Fibroblasts Growing *in vitro*. By J. N. Davidson, I. Leslie and C. Waymouth. (*Biochemistry Department, St Thomas's Hospital Medical School, London, S.E.* 1)

In fresh explants of the 12-day chick embryo heart cultivated in plasma clot in roller tubes (Davidson & Waymouth, 1943, 1945, 1946), the ribonucleic acid phosphorus (RNAP) content is 2–3 times greater than the deoxyribonucleic acid phosphorus (DNAP) content. Cultures incubated for 24–48 hr. in Tyrode solution alone lose as much as 40 % of both types of nucleic acid. The addition of a mixture of embryo extract and serum at this stage is followed by a marked rise in RNAP. Increases in DNAP occur but are less pronounced, with the result that the RNAP : DNAP ratio rises. Regular fluctuations in the RNAP level occur during the first 24 hr. after the addition of growth-promoting media.

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Bilirubin-Serum Protein Complexes. By N. H. Martin (introduced by F. T. G. Prunty). (*St George's Hospital Medical School, London, S.W.* 1)

Bennhold in 1932 demonstrated the migration of bilirubin with the albumin fraction of human serum. The ultracentrifugal studies of Pedersen & Waldenström (1937) seemed to confirm this, and it has, since then, been concluded that the 'bound bilirubin' circulating in the blood stream was attached to the albumin (Watson, 1946).

While studying the changes in electrophoretic pattern of the serum proteins in infective hepatitis (Martin, 1946) it was observed repeatedly that the bilirubin present did not migrate with the same mobility as the albumin. The relationship of bilirubin to the circulating serum proteins was therefore reinvestigated. The methods of fractionation elaborated by Cohn (1946) and his collaborators made it possible to study the behaviour of bilirubin with separate proteins and groups of proteins existing in the human blood stream.

Three approaches were employed:

(*a*) A study of the alteration in the characteristic absorption spectrum of bilirubin in the presence of the protein under test.

(*b*) A study of the migration of bilirubin in a standard electrical field in the presence of the protein under test.

(*c*) A study of the behaviour of the bilirubin in the presence of protein in a two-chambered system the chambers being separated by a semipermeable membrane impermeable to the protein.

Using these techniques it was concluded that bilirubin formed a complex with albumin in a molecular ratio of the order of 2 mol. of bilirubin to 1 mol. of albumin at a pH of 7·4 up to 3 mol. of bilirubin to 1 mol. of albumin observed at a pH of 7·9. This ratio could be altered by previous alteration of amino groups in the albumin molecule, using methyl isourea in a modification of the method of Greenstein (1925).

Interaction also appeared to occur with an α_1 globulin fraction and two α_2 globulin fractions. It did not occur to any significant extent with a purified β globulin nor with an electrophoretically homogeneous γ globulin.

It appears that the present conception must be modified and that under certain conditions in addition to albumin a significant percentage of bilirubin circulating in the blood stream may be bound to the α globulins.

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Folic Acid, B_c Conjugate, B_T and Anti-anaemia Liver Concentrates in the Nutrition of the Mealworm, *Tenebrio molitor*. By G. FRAENKEL. (*Department of Zoology and Applied Entomology, Imperial College, London, S.W. 7*)

We have previously shown (Fraenkel & Blewett, 1947) that mealworm larvae, when grown on an artificial diet, required, in addition to the then known B-vitamins, at least two B-factors, namely, folic acid and another factor, called by us tentatively B_T, which is contained in a charcoal filtrate from yeast or liver extract. In the absence of folic acid growth is greatly retarded, while without B_T the larvae die prematurely. Folic acid (pteroylglutamic acid) can be successfully replaced by vitamin B_c conjugate (pteroylheptaglutamic acid) which has, however, a slightly lesser activity. This is in line with the commonly accepted view that folic acid occurs in natural foods mainly in the form of the conjugate (Welch, Heinle, Nelson & Nelson, 1946). B_c conjugate, on the other hand, has no B_T activity.

Two commercial preparations of anti-pernicious anaemia liver concentrates, manufactured by Lederle Laboratories and Glaxo Laboratories respectively, have been found, in tests with *Tenebrio*, to be entirely devoid of folic acid activity, which indicates the virtual absence of both folic acid and B_c conjugate. These liver extracts also fail to show any B_T activity. This indicates that folic acid, anti-pernicious anaemia factor in liver concentrates and B_T are different substances.

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Some Observations on the Barritt Reaction for the Estimation of Creatine. By A. H. ENNOR and L. A. STOCKEN. (*Department of Biochemistry, Oxford*)

Eggleton, Elsden & Gough (1943) have stated that there are interfering substances present in liver which prevent the estimation of creatine by their modification of the Barritt reaction. Experiments have shown that this reaction is extremely sensitive to inhibition by thiol compounds, and that this inhibition is dependent upon the molar ratio of thiol to creatine. Thus it can be shown that approximately 50% inhibition is produced by a molar ratio of cysteine ester:creatine of 0·6:1. Similar results are given by glutathione (GSH) and benzyl mercaptan.

Mere oxidation of the thiols is insufficient to remove the inhibitory effect because of the lability of the —S—S— bond in alkaline solution. The addition of *p*-chloromercuribenzoate has proved effective for the removal of the inhibition, and by this modification it is now possible to estimate creatine in trichloroacetic acid liver extracts. In view of the general distribution of GSH in tissues it is essential to add the mercury reagent to all extracts in which the molar ratio of thiol : creatine exceeds 0·2 : 1. In the case of small intestine, lung, kidney and spleen the addition has led to increases ranging from 60 to 250%.

It has also been found that the Barritt reaction is inhibited by small amounts of Cu—12·6 μg. of Cu produces a 20% inhibition in the estimation of 26·2 μg. of creatine. This inhibition may be quantitatively removed by the addition of KCN (0·01 M).

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The Effect of Some Thiol Compounds upon Trypsin, Chymotrypsin and Chymotrypsinogen. By R. A. PETERS and R. W. WAKELIN. (*Department of Biochemistry, Oxford*)

In 1943–4, we found that several thiol compounds inactivated crystalline trypsin and chymotrypsin (Kunitz & Northrop, 1935–6); this work was not reported in the stress of events. In the meanwhile, Grob (1946) has published work (also done in 1943) showing that thiol compounds inhibit the activity of crystalline trypsin.

In our experiments, the proteinase activity (as judged by liberation of non-protein nitrogen from casein solutions in 45 min. at 38°) was inhibited almost completely by a previous exposure of the enzymes to the following compounds at pH 7·6 in 0·1 M. concentrations: monothioethylene glycol (MTEG) cysteine (ester HCl) glutathione, BAL, sodium sulphide and *p*-thiosalicylic acid; no inhibition was produced by oxidized MTEG, cystine or pyrophosphate. MTEG and cysteine were also shown to have an action upon chymotrypsinogen. The action was more rapid to the alkaline side of pH 7·0. The thiol effect was irreversible. It was also shown with trypsin, acting upon benzyl arginine amide. Whereas none of the proteins decolorized porphyrindin at pH 7·3, after thiol treatment and removal of thiol, best in acidified acetone (Stern & White, 1937), the proteins decolorized porphyrindin in an amount equivalent to 10–20 % of the total S considered to be present (Kunitz & Northrop, 1935–6; Brand & Kassell, 1941). This is interpreted as a scisson of an —SS— group present in the enzymes upon the lines of the well-known inactivation of insulin (Abel & Geiling, 1925; du Vigneaud, 1927); in this our view differs from that of Grob (1946). It is interesting that three proteins from the pancreas should behave similarly. The action of thiol compounds upon chymotrypsinogen leads to a rapid precipitation at pH 7·6.

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The Possible Importance of Copper in Soil Nitrification. By Howard Lees. (*Rothamsted Experimental Station, Herts*)

The effect of copper sulphate in soil nitrification has been studied in the soil percolation apparatus (Lees, 1947). At a percolate concentration of M/250, copper sulphate inhibited nitrification in three different soils, but at percolate concentrations of M/2000–4000 copper sulphate there appeared to be a faint stimulation. A nitrifying fen soil, treated with sodium diethyldithiocarbamate and thoroughly washed, lost its nitrifying power. This power was restored by the addition of M/250 or M/500 copper sulphate to the percolates but not by the addition of similar concentrations of zinc, cadmium, iron or manganese salts. These results agree with those previously obtained (Lees, 1946), and suggest that copper may be an important trace element in soil nitrification.

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Biosynthesis of the Penicillins. By E. Lester Smith and A. E. Bide. (*Glaxo Laboratories Ltd., Greenford, Middlesex*)

The now general use in penicillin production of phenylacetic acid or related compounds to increase the yield of penicillin G (II or benzyl penicillin) stems from research started independently in America and England. Delay in publication arises from wartime collaboration and secrecy agreements.

The discovery that addition of corn-steep liquor to a 'synthetic' medium induced formation of penicillin G rather than of F (I or Δ^2-pentenyl penicillin), suggested a biosynthetic precursor. DL-β-Phenyl-α-alanine and many of its degradation products were tested, usually in a 'synthetic' medium plus protein hydrolysate under surface culture conditions. β-Phenylethylamine, phenylacetamide, phenylacetic acid, *N*-(phenylacetyl) glycine and benzyl cyanide nearly doubled penicillin yields at optimal concentrations around 0·05 %. Other compounds such as phenylpyruvic acid, phenylacetic acid, β-phenylethyl alcohol and phenylacetaldehyde were less effective even at higher concentrations. Phenylalanine was quite effective but only at high concentrations, e.g. 1 %. The first group were about equally effective on purely synthetic media, and still useful on corn-steep media to supplement the small quantities of precursor present naturally. Several lines of evidence suggested that the phenomenon was due to partial biosynthesis of penicillin G, and not merely to the plant growth-promoting properties of phenylacetic acid and related substances. Phenylacetamide was shown to induce formation of (mainly) penicillin G on a synthetic medium that otherwise yielded penicillin F. Also β-(*p*-fluorophenyl)ethylamine yielded the corresponding *p*-fluorobenzylpenicillin.

The formation of small proportions of penicillin X (III or *p*-hydroxybenzylpenicillin) was presumably induced by a corresponding precursor present in corn-steep liquor. Penicillin X formation was markedly suppressed by both phenylacetic acid and phenylethylamine.

The use of supplements designed to induce biosynthesis of penicillins with aliphatic side-chains failed to increase the yield of total penicillin.

Many substances that might function as intermediates for other parts of the molecule, also had little or no effect on penicillin yields. More complex precursors such as *N*-(phenylacetyl)- and *N*-(β-phenylethyl)-amino-acids were used with the idea that they might be incorporated nearly intact into the penicillin molecule. Later evidence indicated that effective compounds were split by the mould, only the benzyl group appearing in the penicillin. A sulphur-free medium permitted only negligible mould growth. Out of a number of inorganic and organic sulphur compounds, sulphate was most effective in stimulating growth and penicillin production.

Penicillin Precursors in Corn-steep Liquor. By T. H. MEAD and M. V. STACK. (*The British Drug Houses Ltd., London*)

It was discovered by American workers that the addition of about 8 % of corn-steep liquor (C.S.L.) to the medium on which *Penicillium notatum* is grown causes a fourfold increase in the yield of penicillin and a reduction in the necessary period of incubation. In view of the consequent importance of C.S.L. and because no other substance having similar properties was known, this investigation was undertaken in 1944 as part of the penicillin research programme of the Therapeutic Research Corporation.

A study of the effects of adding to simple media, C.S.L. and fractions prepared therefrom indicated that the activity of the liquor is due to (i) mould growth stimulants, (ii) specific penicillinogenic factors. Difficulties encountered in assaying the specific factors in fractions of C.S.L. from which the non-specific growth stimulants had been removed, were overcome by using negative control cultures grown on a basic medium containing casein hydrolysate as growth promotor and by adding sufficient casein hydrolysate to the experimental cultures to bring their total nitrogen concentration up to those of the negative controls and of positive controls containing C.S.L.

With the aid of this assay it was shown that the specific penicillinogens of C.S.L. migrate to the cathode compartment on electrodialysis, are lost by evaporation of alkaline but not acid solutions, and form insoluble phosphotungstates from which they are recoverable in the usual manner. Since many benzyl compounds are effective penicillinogens (Lester Smith & Bide, 1948), the presence of β-phenylethylamine was suspected. This base was finally isolated from C.S.L., made alkaline with K_2CO_3, by vacuum-steam distillation. It was purified by ether extraction and recovery from the ether by aqueous acid and was separated from much accompanying ammonia by extraction of the mixed hydrochlorides with chloroform. Phenylethylamine hydrochloride was identified by analysis and mixed melting-point and conversion to the benzoyl derivative. The yield, probably far less than quantitative, was 0·017 %—sufficient to provide the benzyl component of 75 units of penicillin/ml. assuming the medium to contain 10 % of C.S.L.

β-*p*-Hydroxyphenylethylamine was isolated in 0·018 % yield by electrodialysis followed by solvent extraction. Its identity was established by analysis and mixed melting-point of the hydrochloride, free base and dibenzoyl derivative.

Extracts of unfermented maize were inactive. The bases isolated may arise from bacterial decarboxylation of the corresponding amino-acids during the steeping process. Changes in the bacterial population of the steeping vats may account for some of the variations in penicillinogenic activity displayed by different batches of C.S.L.

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The Biosynthesis of Penicillins by *Penicillium notatum*. By H. R. V. ARNSTEIN, J. R. CATCH and A. H. COOK. (*Organic Chemistry Department, Imperial College, London, S.W. 7*)

The increase in penicillin production caused by adding certain supplements to the nutrient medium on which *P. notatum* was cultured suggested that these supplements might be incorporated directly into the resulting penicillin. Accordingly, a strain was selected and grown under conditions which, in absence of the chosen supplement (phenylacetamide), afforded mainly Δ^2-pentenylpenicillin (penicillin I or F) and no significant yield of benzylpenicillin (penicillin II or G). Chromatography of the material obtained on the supplemented medium then showed it to contain a considerable proportion of benzylpenicillin, the identity of which was left in no doubt by its conversion into the characteristic benzylpenillic acid.

A perhaps more stringent examination of the overall role of the supplement was provided by the chromatographic analysis of penicillin produced by a strain of *P. notatum* in presence of *p*-hydroxyphenylacetic acid. This penicillin mixture was found by indubitable chemical means to contain *c.* 35 % of *p*-hydroxybenzylpenicillin (penicillin III or X) as compared with *c.* 5 % of this variety of the antibiotic when grown without the supplementary nutrient.

These biosyntheses demonstrated some measure of control over the nature as well as the gross amount of the penicillin produced. The possibility of biosynthesizing 'unnatural' penicillins was thus envisaged and has since been realized elsewhere.

The Effect of Adrenal Cortical Hormone *in vitro* on Carbohydrate Synthesis in Liver Slices.

By Chiung-Yun Chiu and D. M. Needham. (*Biochemical Laboratory, University of Cambridge*)

The observation of Seckel (1940) that addition of adrenal cortical hormone (eschatin) *in vitro* causes inhibition of glycogenolysis in liver slices, has been confirmed. It was further found that if the experiments were done in nitrogen instead of in air the eschatin was without effect on glycogenolysis. This observation showed that the action of eschatin is on glycogen synthesis rather than on glycogenolysis, and in the present work conditions favourable to synthesis were used.

The experiments were carried out in Barcroft pots filled with oxygen, the slices (about 100 mg.) being suspended in 3 c.c. of bicarbonate Ringer's solution buffered with M/150 phosphate, pH 7·4. The concentration of eschatin (kindly supplied by Messrs Parke Davis without preservatives) giving maximal effect after 1 hr. incubation at 37° was 6 rat units in 3 c.c.

Some typical results are shown in the table.

Since eschatin seems to favour glycogen formation from all the substrates tested it seems probable that it affects some common reaction such as one of those concerned in continual oxidative resynthesis of ATP. This is under investigation.

	Glycogen (mg./g.)		
Substrate	Initial	Final + eschatin	Final no eschatin
	Rat liver slices		
0·45 % D-lactate	0·76	1·75	1·06
0·45 % Na-pyruvate	1·77	4·32	1·92
1 % glucose	—	—	—
0·4 % DL-alanine	—	—	—
	Rabbit liver slices		
0·45 % D-lactate	—	—	—
0·45 % Na-pyruvate	—	—	—
1 % glucose	6·39	13·20	11·00
0·4 % DL-alanine	1·09	1·18	0·79

In experiments in oxygen with no added substrate, there was either glycogen disappearance or a small increase in the slices without eschatin. When eschatin was added the balance was turned in favour of synthesis; this was shown either by a decrease in glycogen disappearance or a greater increase in glycogen content. Since it seems likely that, in the absence of added substrate, protein is the source of the glycogen formed, the effect of eschatin on non-protein nitrogen formation was tested. About 20 % more non-protein nitrogen was found in the presence of eschatin in each case at the end of the experiment.

In four experiments on rat liver slices with pure desoxycorticosterone acetate (DOC) it was found that 10 μg. caused marked increase in glycogen content: for example (*a*) with pyruvate as substrate, initial value 1·31 mg./g., final with DOC 3·05; without DOC 1·37; (*b*) with D-lactate, initial value 1·34 mg./g., final value with DOC 1·85; without DOC 1·40.

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The Hexokinase-inhibiting Activity of Diabetogenic Pituitary Extracts.

By E. Reid (Carnegie Research Fellow), R. H. Smith and F. G. Young. (*Department of Biochemistry, University College, London*)

The important discovery of C. F. Cori and his colleagues (Price, Cori, C. F. & Colowick, 1945; Price, Slein, Colowick & Cori, G. T., 1946; Cori, C. F., 1946; Colowick, Cori, G. T. & Slein, 1947) that the transfer of phosphate from adenosine triphosphate to glucose under the influence of the enzyme hexokinase is inhibited *in vitro* by anterior pituitary extracts, and that this inhibition is counteracted by the addition of insulin to the system *in vitro*, has led many to the assumption that the pituitary substance concerned is the diabetogenic factor. The discoverers themselves do not claim that this is so, and from their own demonstration of the extreme lability of the pituitary hexokinase-inhibiting factor it may be concluded that there is no simple identity established between this factor and the pituitary diabetogenic substance, since in our hands the latter remains stable at 0° for many weeks under suitable conditions and may even survive long periods at room temperature (Young, 1946).

In a number of instances our experiments have confirmed Cori's essential findings, including the reversal by insulin of pituitary hexokinase inhibition and the lability of the pituitary factor concerned. Furthermore, we have failed to find an insulin-reversible hexokinase inhibitory activity in many anterior pituitary preparations which are highly diabetogenic in the adult intact cat, including, in some instances, fractions prepared according to the methods of Cori *et al.* for the production of the pituitary hexokinase inhibition. These experiments have been carried out with pituitary extracts free from added antiseptic. In other pituitary preparations we have had present 0·01 % sodium ethyl mercurithiosalicylate ('Merthiolate'-Eli Lilly); these have often exhibited hexokinase inhibitory activity *in vitro* but in a manner which is not significantly reversible by insulin in our hands. Moreover, the factor here concerned appears to be stable at 0°.

Our results demonstrate a wide divergence between the insulin-reversible hexokinase inhibitory activity and the diabetogenic activity of anterior pituitary extracts. Nevertheless, in agreement with Cori, we have observed that when crude anterior pituitary extracts (in our experiments possessing high diabetogenic activity but no demonstrable insulin-reversible activity on hexokinase *in vitro*) are administered to intact rats the muscle hexokinase activity is influenced in such a way that it can be enhanced by the addition of insulin *in vitro*. It may well be that the insulin-reversible hexokinase inhibitor and the diabetogenic factor of anterior pituitary extracts are substantially the same but that in stored pituitary extracts the factor undergoes a rapid change which deprives it of its insulin-reversible inhibitory activity *in vitro*, such a change being reversible *in vivo*.

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The Hexokinase Activity of Retinal Extracts.

By MARGARET KERLY. (*Department of Biochemistry, University College, London*)

The high glucolytic activity of aqueous extracts of ox retina suggested an investigation of their hexokinase activity. Extracts were prepared according to our previous technique (Kerly & Bourne, 1940); they contained negligible amounts of glucose or of adenosine triphosphate (ATP). Hexokinase activity was tested by incubation in a bicarbonate buffer containing Mg^{++}, NaF and an excess of glucose and of ATP. In crude extracts, even in the presence of a high concentration of fluoride, glucose-6-phosphate formed by the reaction of glucose and ATP undergoes further change. Most of the labile phosphate transferred from the ATP accumulates as an ester not readily hydrolyzed by 1·0 N-HCl, but an appreciable amount of an easily hydrolyzable ester is also formed. This complicates estimation of the hexokinase activity either by determination of loss of ATP or by manometric measurement, and it is preferable to determine glucose utilization. When 0·1 ml. extract is incubated at 30° for 10 min. about 200 μg. glucose is removed. In these conditions the rate of the reaction is nearly linear and varies with the amount of extract added. Fructose is utilized at a rather more rapid rate; neither 3-methylglucose nor galactose is attacked.

These extracts retain their hexokinase activity for several days when stored in the cold room. Dialysis for short periods (up to 24 hr.) has little effect on activity, but after 72 hr. activity is slight.

Preliminary attempts at purification using the methods of Cori & Slein (1947) have shown that an active extract can be recovered from precipitates formed on addition of acetone or ammonium sulphate to crude extracts.

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The Effect of Adrenaline Injection on Glucose Utilization by Muscle.

By J. A. COHEN and DOROTHY M. NEEDHAM. (*School of Biochemistry, University of Cambridge*)

The possibility that the hyperglycaemia following adrenaline administration might be partly due to a decrease in peripheral glucose utilization has been the subject of controversy. The experimental evidence was all derived from intact or eviscerated animals, and one of us (1947) has recently tried to throw some light on the problem by experiments *in vitro*. It was shown that injection of adrenaline into rats did lead to a partial suppression of the anaerobic glycolysis in diaphragm. The results suggested that this effect was an indirect one, mediated by other hormones possibly of hypophyseal or adrenal cortical origin.

In the present series of experiments we have tried to elucidate this effect on muscle by investigating the glucose utilization of fresh rat muscle extracts. Such extracts from normal animals, under our conditions (0·2 % glucose, 0·007 M-ATP) utilize glucose in the course of 40 min. at 30° to the extent of about 2·3 mg./ml. extract. Extracts made in exactly similar manner from litter mates injected 45 min. previously with a dose of adrenaline (1 mg./kg.) showed a glucose utilization decreased by 29 %. These results are the average from experiments on 19 normal and 19 adrenaline injected animals.

This inhibition was also found in experiments of short duration (10 min.). In these, the glucose concentration was 0·05 % and the ATP concentration 0·005 M. The glucose utilization under these conditions was about 0·7 mg./ml. extract in the extracts from normal animals and 28 % less in the extracts from injected animals. Thirty animals were used in each case for obtaining these averages. By adding fluoride to the system (0·06 M) the ATP can be maintained constant in the short-term experiments (since ATP-ase is inhibited) and under these conditions the glucose utilization may be regarded as a measure of hexokinase activity. An inhibition of 13 % was found, suggesting that at least part of the inhibition in the absence of fluoride is due to an effect on hexokinase. As in the case of diaphragm, it was not possible to show any direct action of adrenaline on muscle extract; this indicates an indirect action, e.g. through other hormones liberated as a result of adrenaline injection or through products resulting from adrenaline metabolism. One of these products, adrenochrome, was found not to have any inhibitory effect in short term experiments.

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The β-Glucuronidase Activity of Ox Spleen.

By G. T. MILLS. (*Biochemistry Department, St Thomas's Hospital Medical School, London, S.E.* 1)

The data of previous workers on β-glucuronidase (Masamune, 1934; Oshima, 1936; Fishman, 1939; Graham, 1946; Talalay, Fishman & Huggins, 1946) indicated the presence of only one enzyme in spleen and kidney which hydrolyzed β-glucuronides. The pH optima for these preparations have been stated to be 5·0–5·3 for L-menthylglucuronide (Oshima, 1936; Fishman, 1939), 5·3–5·6 for phenylglucuronide (Oshima, 1936) and 4·5 for phenolphthalein glucuronide (Talalay *et al.* 1946).

A reinvestigation of the preparation and purification of β-glucuronidase, involving the application of the variable solvent solubility test, has shown the presence in ox spleen of two enzymes hydrolyzing certain β-glucuronides. These two β-glucuronidases have been separated and purified by ammonium sulphate fractionation and examined separately.

The pH optima for the hydrolysis of L-menthylglucuronide are 4·5 and 5·0 for these preparations.

The preparative methods of previous workers were such as to eliminate the enzyme having the lower optimum pH.

Data are presented on the hydrolysis of β-glucuronides other than D-menthylglucuronide by the two separate enzymes.

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Pathologic Effects of Ionizing Radiations and Radioactive Materials. By A. M. Brues (introduced by Sir E. Mellanby). (*Argonne National Laboratory, Chicago, Illinois*)

Certain purely chemical effects of ionizing radiations are understood. These include the dissociation of water into H and OH radicals and the inhibition of certain enzymes such as those containing sulphydryl groups (Dale, 1942; Barron, 1947). In addition to these proximate effects, various forms of genetic and cell damage have been extensively studied. The relation of these changes to most clinical radiation effects remains largely to be established.

In acute lethal radiation exposure in mammals, we see an immediate response corresponding to clinical X-ray sickness. Susceptible animals will show hypotension, vomiting, or erythema of the skin (Painter, Prosser & Moore, 1947). By the second day, acute symptoms have largely abated, but there is a marked decrease in circulating white cells which continues until death. During the ensuing week, animals refuse food and water and lose weight. There is increasing anaemia, increased plasma and extracellular volume, accelerated sedimentation rate, increased renal excretion of phenol red, and decreased levels of blood non-protein nitrogen, urea and polypeptides. Histologic damage is most marked in the blood-forming organs, gonads, and intestinal tract.

During the second week, the clotting time is greatly increased due to an overabundance of circulating heparin (Allen & Jacobson, 1947); fever and subcutaneous oedema appear and death occurs, attended by loss of blood, bleeding into vital areas, such as the heart and central nervous system, ulceration of mucous membranes and a terminal shock-like state. A shift in the plasma protein pattern (increased beta and gamma globulin) has been seen in dogs, but is not specific for radiation sickness (Prosser, Painter & Swift, 1947).

Later effects in surviving animals include anaemia, lymphoid atrophy, and in mice, an enhanced tendency to lymphoma. Chronic effects include decrease in life span, greying of hair and an increased tumour incidence.

Acute effects of absorbed radio elements generally resemble those of total body radiation. Their late effects depend upon localization of the element and, as in the case of radium, a great increase in bone tumours is seen after absorption of radiostrontium, as well as plutonium and many of the radioactive rare earths (Lisco, Finkel & Brues, 1947).

Since C^{14} has been shown to deposit in bone (Bloom, Curtis & McLean, 1947), the question is raised whether it may present a human hazard analogous to radium. An attempt has been made to consider the human tolerance on the basis of known facts and it is presently thought that a total human absorption in excess of 30 microcuries must be considered hazardous until further knowledge is gained regarding the retention of carbon administered in various forms. In the case of a hypothetical carbon compound, such as a steroid, which might remain localized in a very small organ, even this figure may need revision.

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DEMONSTRATIONS

A Simple Automatic Soil Percolator. By HOWARD LEES

An apparatus that continually percolates a column of soil with small quantities of metabolite solution from a reservoir, to which they are returned after percolation. The apparatus, which has already been described (Lees, 1947), is a modified and improved version of my original one (Lees & Quastel, 1944). Any weight of soil up to 40 g. of any percolate volume between 50 and 200 ml. may be used. The apparatus is driven equally well by air pressure or by suction, is in a large measure self-adjusting in operation, is quickly assembled or disassembled, and is simply constructed, there being only two glass joints. At no point in it does the soil or percolate touch any material save glass. The soil cannot become waterlogged because if air flow through the soil becomes impeded, percolation automatically stops.

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An Apparatus for the Preparative Electrophoretic Separation of Amino-acids, Peptides and Proteins. By J. A. V. BUTLER and JOAN M. L. STEPHEN

A Simple Apparatus for the Extraction of Corticosteroid-like Substances from Urine. By ALICE M. ROBINSON and F. L. WARREN. (*Pathology Department, St Bartholomew's Hospital, E.C. 1, and Physiology Department, St Mary's Hospital Medical School, London, W. 2*)

Several procedures have been devised for the assay of 'corticosteroid-like' substances in human urine by chemical methods. The initial step usually consists in the extraction of urine (either fresh or after acidification) with chloroform. Normal methods of extraction by hand-shaking in separatory funnels frequently lead to troublesome emulsions and partial loss.

The formation of these emulsions is avoided by the exclusion of air during admixture of the two liquids. In the present type of apparatus this is achieved by delivering the urine in the form of a fine spray at the bottom of a column of chloroform. The urine rises through the chloroform in droplet form and coalesces as an upper aqueous layer which is removed via a side outlet and recirculated through the chloroform.

In an extraction apparatus of appropriate size the whole of the 'corticosteroid-like' substances in 100 ml. of urine can be transferred to chloroform in 50–70 min. (ten recirculations) and this amount of extract, after further purification, is sufficient for chemical assay.

Apart from the ease and semi-automatic nature of the extraction, a further advantage is the increased speed (compared with hand-extraction) which is an important factor in obtaining maximum yields in view of the instability of the 'corticosteroid-like' substances.

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from the author.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **262nd Meeting** *of the Biochemical Society was held in the London School of Hygiene, Keppel Street, London, W.C. 1, on Saturday, 13 December 1947 at 11 a.m., and took the form of a Symposium on 'The Biochemical Reactions of Chemical Warfare Agents'.*

The Reactions of Mustard Gas in Aqueous Solution. By A. G. Ogston. (*Department of Biochemistry, Oxford*)

An effort is made to trace the history of research carried out during the years 1939–45 on the replacement reactions of mustard gas in aqueous solution, in Britain and in the U.S.A. and to consider the results in relation to the actions of mustard gas on biological systems. Starting from the pioneer kinetic work of Peters & Walker (1923), kinetic investigation showed that the reaction of mustard gas is of the 'SN_1' type described by Ingold: an initial first-order reaction gives rise to an activated form of mustard gas, which then undergoes second-order reactions with a wide variety of reagents. These secondary reactions compete with each other, so that the final products depend on the relative concentrations and velocity constants of reaction of the substituting reagents present in the system. Later work filled in and refined this simple account, taking into consideration the effects on the kinetics of reaction of the presence of two reactive chlorine atoms in mustard gas, of varying ionic strength and of the reversibility of formation of some of the products of reaction, particularly those involving the formation of sulphonium compounds between mustard gas and other sulphides.

It appeared from these investigations that mustard gas can combine with all the chemically reactive groups found in biological systems. Direct investigations, especially on proteins and enzymes, gave results in full accord. Certain remaining problems concerning the toxic effects of mustard gas are discussed.

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Reactions of Mustard Gas with Enzymes in Tissues and *in vitro*. By D. M. Needham. (*Biochemical Laboratory, University of Cambridge*)

Mustard gas (H) is not a general enzyme poison. Out of some 30 enzymes examined only those of two well-defined groups are markedly inhibited—the phosphokinases and closely related enzymes and a small group of proteinases.

Amongst the phosphokinases, hexokinase was the first to be examined and found to be H-sensitive. It was shown to be an —SH enzyme, but the inhibitory effect of H is not through the —SH group. Pure crystalline hexokinase was prepared from yeast in several laboratories; its reaction with H containing radioactive sulphur was examined by Bailey and Webb, who came to the conclusion that only 5–6 molecules of H are necessary per molecule of hexokinase for complete inactivation.

It was soon found that other phosphokinases are H-sensitive, e.g. creatine-, phosphopyruvic-, and glycerol phosphokinases and deuterohexokinase. The phosphorylation connected with oxidations in extracts is also inhibited by H, although the oxygen uptake is not.

The proteinases inhibited by H are pure pepsin (Northrop), a new tissue peptidase (Bergmann) and a new skin proteinase (Peters).

The work concerned with the effect of H on enzymes in tissues has dealt mainly with skin, marrow, intestinal and gastric mucosa and muscle. In this field it is often necessary to consider whether the effects observed are due to the action of H itself or to the action of products formed in the tissues.

Some Reactions of Mustard Gas (ββ′-Dichlorodiethyl sulphide) with Proteins. By J. C. Boursnell. (*School of Biochemistry, University of Cambridge*)

In this paper a short account was given of the action at room temperature and at 37°, of mustard gas (*H*) and some of its derivatives upon a variety of proteins.

Some indication was given of the extent and firmness of the combination which takes place, and also of some of the groups in the protein molecule which are most probably attacked. In a number of these experiments use was made of radioactive sulphur (S^{35}) as a tracer element.

Attention was also given to some immunological investigations using the reactions of mustard gas (and some of its derivatives) with serum proteins to produce protein derivatives with a new immunological specificity; and reference was made to the inactivating action of mustard gas and some of its derivatives upon complement.

The Development of British Anti-lewisite. By R. H. S. Thompson. (*Department of Chemical Pathology, Guy's Hospital Medical School*)

The view that the toxic action of arsenoxides on living cells involves a reaction with thiol groups is not new. Voegtlin and his colleagues (1923, 1925) and Peters & Walker (1924) had each demonstrated the limited ability of certain monothiols to cause some degree of protection of certain biological systems from intoxication by therapeutic and other arsenicals. The effects obtained were, however, much less complete when exerted against sodium arsenite, and when, during the last war, monothiols were tested against lewisite no significant degree of protection could be demonstrated.

A re-examination of the problem indicated that the mode of action of small concentrations of arsenic is selective. Of a number of different enzymes examined the pyruvate oxidase system was shown to be the most sensitive, and both *in vitro* and *in vivo* evidence was obtained suggesting that the first point of attack of arsenicals is on pyruvate oxidation. In their actions on enzymes arsenicals in fact resemble iodoacetate, a known reactor with thiol groups.

To study this reaction further, arsenical derivatives of kerateine were prepared, analysis of which showed that arsenic was present largely in the proportion of 1 atom of arsenic to 2 SH groups in the original kerateine. In the hope of forming a ring compound with the arsenical at least as stable as that formed with the tissue 'acceptor' dithiol compounds were therefore studied as potential antidotes. 2,3-Dimercaptopropanol (British Anti-Lewisite) was synthesized (Stocken, 1947) and found to be an efficient antidote when tested *in vitro* with the pyruvate oxidase enzyme system, and *in vivo* against lewisite intoxication or contamination in rats, guinea-pigs and man (Stocken & Thompson, 1946).

From this work with arsenicals and thiol antidotes, evidence has been obtained in favour of the view that, in addition to 'monothiol' enzymes, 'dithiol' enzymes may exist, which, like the pyruvate oxidase system, require the presence within their molecule of 2 closely placed SH groups.

Reactions of Lachrymators with Enzymes and Proteins. By M. Dixon. (*School of Biochemistry, University of Cambridge*)

A survey of the action of lachrymators on enzymes (Mackworth, 1940–1) showed that they act as powerful poisons of —SH enzymes. All the lachrymators, whatever their chemical structure, share this common property. All the —SH enzymes are poisoned by them; other enzymes are not affected, nor are the oxidized forms of the —SH enzymes. This suggested that they react specifically with —SH groups in proteins, and direct —SH tests (Morgan, 1942) confirmed this. Masking the —SH groups by reversible combination with arsenicals protects the enzyme against attack by lachrymators (van Heyningen, 1942). The lachrymators form valuable specific reagents for identifying —SH enzymes.

The '—SH theory' provides for the first time an explanation for the existence of two main groups of lachrymators: (*a*) substances containing a halogen atom in some such group as —CH_2Cl, together with a neighbouring keto or other group which makes the halogen 'positive', (*b*) substances containing a —CH=CH— group, also with a neighbouring keto or other group which polarizes the double bond. Both these types of molecules readily combine with —SH groups.

Lachrymators of type (*a*) can be used for manometric estimation of —SH groups in proteins. With heat-denatured ovalbumin the values obtained agree with those given by other methods, indicating that the protein contains no other groups which react with the lachrymator. Native ovalbumin gives no immediate reaction, but a slow reaction occurs, which ultimately reaches about the same value and is probably due to a slow production of —SH groups. The product, however, does not appear to be denatured.

The Reaction of Fluorophosphonates with Esterases. By E. C. Webb. (*Biochemical Laboratory, University of Cambridge*)

The early studies on the fluorophosphonates prepared by Dr B. C. Saunders had shown that they were not only highly toxic, but showed marked sympatheticomimetic actions in sub-lethal concentration, similar (except in time relations) to those produced by the alkaloid eserine. It was demonstrated independently in 1941 by Adrian & Feldberg and by Mackworth, that serum cholinesterase is inhibited by alkyl fluorophosphonates, as well as by eserine.

The inhibition by eserine reaches a maximum within a few minutes, can be reversed by dialysis, and shows substrate competition; while that by fluorophosphonate is non-competitive and cannot be reversed by dialysis. The time course of inhibition by fluorophosphonate shows two stages. Recently, Nachmansohn, Rothenberg & Feld (1947) have shown that this inhibition can be partially reversed by dilution in the early stages.

Horse serum cholinesterase is 50% inhibited in 15 min. at 20° under standard conditions by $1\cdot3\times10^{-9}$ M-di*iso*propyl fluorophosphonate (the most active ester examined); the inhibition is complete at 10^{-8} M, and still measurable at 10^{-11} M. The figures, taken in conjunction with the absolute activities obtained by Easson & Stedman (1936) and Straus & Goldstein (1943), suggest that complete inactivation can be produced by the presence of 1–3 molecules of fluorophosphonate/enzyme active centre.

Certain other esterases, especially liver esterase and human milk lipase, are inhibited by fluorophosphonate. The pattern of sensitivity to fluorophosphonate of a range of esterases is different from that to eserine or to fluoride, but similar to the pattern of sensitivity to tri-*o*-cresyl phosphate and tri-*o*-chlorophenyl phosphate described by Bloch (1943) and Hottinger & Bloch (1943).

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Pharmacology of Chlorethylamines. By E. Boyland. (*The Chester Beatty Research Institute, The Royal Cancer Hospital* (*Free*), *London, S.W.* 3)

Simple aliphatic chlorethylamines with more than one chlorethyl group are vesicants and many of their pathological effects are similar to those produced by vesicants containing sulphur or arsenic. Compounds with only one chlorethyl group such as dimethyl β-chlorethylamine are much less toxic but have a prolonged neurotoxic action causing tremor and incoordination of movement. Mice treated with such compounds resemble waltzing mice in their behaviour. Dibenzylchlorethylamine blocks and reverses the vasopressor response to adrenaline.

The bis-(β-chloroethyl)-amines with simple alkyl groups, the tris-(β-chloroethyl)-amines and the simple tetrakis-(β-chloroethyl)-diamines are radiomimetic poisons like mustard gas. The effects which they produce are similar to those following treatment with X-rays or radioactive materials. These effects include (1) inhibition of mitosis and fragmentation of chromosomes in plants and animals, (2) mutations in *Drosophila*, *Neurospora* and *Escherichia coli*, (3) nausea and vomiting in human subjects, (4) greying of hair, (5) reduction of the concentration of leucocytes and thrombocytes in the blood, (6) haemoconcentration in dogs, and (7) death of animals after 6 to 10 days. Poly-(β-chloroethyl)-amines have been used in place of radiation therapy in treatment of leukaemia, Hodgkin's disease and bronchogenic carcinoma.

The poly-(β-chlorethyl)-amines react rapidly with water in neutral solution to give chlorethyl ethylene imines. These compounds react further to give hydroxyethyl ethylene imines which are toxic substances killing animals very rapidly following subcutaneous injection.

The **263rd Meeting** *of the Biochemical Society was held at the British Postgraduate Medical School, Ducane Road, Hammersmith, London, W. 12, on Saturday, 24 January 1948, at 11 a.m., when the following papers were read:*

COMMUNICATIONS

The Micro-estimation of Amino-nitrogen and its Application to Paper Partition Chromatography.

By A. J. Woiwod. (*Wellcome Physiological Research Laboratories, Beckenham, Kent*)

A copper phosphate reagent has been developed for determining 1–30 μg. α-amino-N, based on the copper reagent of Pope & Stevens (1939). The amino compound reacts with the copper phosphate to give a soluble copper complex which is quantitatively decomposed by sodium diethyl-dithio-carbamate. The yellow colour formed is extracted into amyl alcohol, and its optical density determined with the Spekker absorptiometer. The amount of copper may be related to α-amino-N by the use of standard curves. The ratio α-amino-N/copper for a number of amino-acids and related compounds is given below. For the formation of the complex A_2Cu the theoretical ratio should be 0·44.

To prepare the reagent, 0·16 M-$CuCl_2 . 2H_2O$ (1 vol.) is added to Na_3PO_4 solution (1 vol.) prepared as described by Pope & Stevens (1939). The copper phosphate sludge formed (1 vol.) is then refluxed for 30 min. with 0·18 M-$Na_2HPO_4 . 12H_2O$ (2 vols.). This reagent is stable for some weeks. The amino-acid solution is pipetted into 0·18 M-$Na_2HPO_4 . 12H_2O$ containing $Na_2B_4O_7 . 10H_2O$ (4·0 g./l.) and an equal volume of the copper phosphate reagent added. After standing 30 min. at 20° C. the mixture is filtered and the copper in the filtrate estimated absorptiometrically.

The method has been successfully applied to the quantitative estimation of amino-acids separated by paper partition chromatography (Consden, Gordon & Martin, 1944).

	Ratio $\frac{\alpha\text{-amino-N}}{\text{Cu}}$		Ratio $\frac{\alpha\text{-amino-N}}{\text{Cu}}$
Alanyl-glycine	0·22	Tyrosine	0·43
Histidine	0·25	Arginine	0·44
Hydroxyproline	0·38	Phenylalanine	0·45
Aspartic acid	0·39	Cystine	0·45
Serine	0·40	Tryptophan	0·46
Threonine	0·40	Glutamine	0·46
Valine	0·41	Alanine	0·46
Leucine	0·41	Proline	0·48
*iso*Leucine	0·41	Lysine	0·52
Glutamic acid	0·42	Ornithine	0·58
Methionine	0·42	Glucosamine	1·11
Asparagine	0·43	β-Alanine	6·89
Glycine	0·43		

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The Inhibition of Acid Phosphatase by D-Tartrate.

By M. A. M. Abul-Fadl and E. J. King. (*Postgraduate Medical School of London, W. 12*)

Amongst the organic substances examined by Kutscher & Wörner (1936), alcohols, especially the lower aliphatic homologues and certain narcotics, were found to exhibit strong inhibitory action on the prostate enzyme. King, Wood & Delory (1945) also pointed out that the red cell phosphatase was destroyed by ethanol. The effect of formaldehyde has been discussed by the authors in a previous communication (1947). The effect of certain organic acids has now been investigated with regard to their action on normal plasma, prostate, and red cell acid phosphatases. Oxalates were found to inhibit all of them, nearly to the same extent. Bile acids inhibited almost equally the red cell and prostate enzymes, but did not affect the acid phosphatase of normal plasma.

Tartrates were without noticeable effect on either the plasma or the second (pH 5) red cell phosphatase, but, on the other hand, were very strongly inhibitory toward the prostate enzyme. It was thought that the chemical configuration of this acid might be involved; and a variety of related hydroxy and other carboxylic acids were examined (formic, acetic, lactic, pyruvic, malic, succinic, citric and salicylic acids). None of these was found to exhibit the effect shown so strongly by tartaric. With the different stereo-isomerides of tartaric acid, the property of inhibition was only possessed by the D- and DL-forms, while the L- and the *meso*-isomerides were ineffective. Thus the tartrate inhibition can be considered to be stereomeric. It sharply distinguishes the plasma and red cell phosphatase on the one hand, from the prostate and other tissue phosphatases on the other.

Like the competitive type of inhibition to which the oxalate and possibly the fluoride inhibitions belong, this D-tartrate inhibition can be reversed by dialysis. It is distinct from the destructive type of inhibitor, e.g. by ethanol and formaldehyde.

The effect of D-tartrate on the other tissue phosphatases at various pH values was also studied. It has been found that the liver, kidney and spleen enzymes are also strongly inhibited in the acid range, while not affected in the alkaline. The red cell phosphatases were inhibited in the acid range of pH 3·8–4·6, while from pH 4·8 upwards there was either no inhibition or slight activation. This favours the presence of two acid phosphatases in erythrocytes, as found by Roche, Thoai & Baudoin (1942), and confirmed by ourselves.

The application of tartrate in the determination of serum acid phosphatase for clinical purposes is being investigated.

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Bilirubin: Modified Procedure without Precipitation of Proteins. By R. V. Coxon and E. J. King

The Apparent Arginine Content of Human Plasma Proteins by the Sakaguchi Reaction. By J. W. Keyser

A New Study of Protein Digestion (in Dogs). By C. E. Dent* and J. A. Schilling. (*Departments of Pathology and of Surgery, University of Rochester, New York*)

Doubt still exists as to whether the substances absorbed into the portal blood during protein digestion are mainly amino-acids or peptides (Verzar & McDougall, 1936). The existing evidence suggests that absorption of all, even also of traces of intact protein, may occur to some extent.

The paper chromatographic technique (Consden, Gordon & Martin, 1944) has now been applied to the problem. Some 60 amino-acids or closely related compounds likely to occur in nature have been studied on the chromatograms and the method is confirmed to be highly specific qualitatively and also to allow of rough quantitative comparisons. Portal blood, drawn by means of the London cannula (London, 1936), and in some cases also jugular blood and urine, have been examined at 1, 2·5 and 5 hr. intervals after feeding various proteins. In nearly every case the dog was previously submitted to a partial gastrectomy operation. This enabled the protein to pass more rapidly from the stomach into the small intestine. The ensuing quickened digestion resulted in much larger rises (4–6 times normal) of the levels of the metabolites in the portal blood, and the peak of absorption was always within the 5 hr. span of the experiment. The plasma proteins were removed by ultrafiltration before the chromatographic analysis.

It has been shown in this way that casein, casein hydrolysate (Amigen), ground beef and human serum albumin are all absorbed mainly, if not entirely, as free amino-acids. No peptides were found in the blood although some samples showed, after acid hydrolysis, slight rises in certain amino-acids. The urine after feeding human albumin did, however, show the presence of 'peptides', i.e. ninhydrin-reacting substances, moving characteristically on the chromatograms and readily decomposable by hydrolysis.

In marked contrast to the above results, there were no appreciable changes in blood amino-acid levels after feeding dog's plasma protein although the protein was shown to have disappeared from the gut. There was also a tendency to diarrhoea and vomiting in these dogs. This was not observed with the other proteins.

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The changes in free and bound plasma amino-N in several of the experiments have been kindly checked by Dr H. N. Christensen, using the ninhydrin-CO_2 method.

The suggestion is made that the dog's plasma protein was absorbed intact under the conditions of the experiment. The dog's intestinal mucous membrane appears therefore to be able to distinguish its own plasma proteins from a human plasma protein as well as from the beef and casein. This interpretation of the facts leads, however, to serious difficulties in reconciling other work in which homologous plasma was fed.

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The Relative Importance of Antibodies and Vitamin A in Preventing Disease in Young Calves.

By F. Blakemore, A. W. Davies, Eva Eylenburg, T. Moore, K. C. Sellers and A. N. Worden. (*Institute of Animal Pathology and Dunn Nutritional Laboratory, Cambridge*)

Exploratory experiments confirmed previous claims that calves develop fatal 'white scour' when they are denied colostrum and kept on a diet of whole or separated milk. Ayrshire and Jersey calves were found to be particularly susceptible to the disease, while Friesians, which are larger in size, were more resistant.

The protective action of colostrum has been ascribed to its antibody content, but since the calf is normally born with low reserves of vitamin A its significance as a rich source of this vitamin has also been considered. Attempts to protect calves from 'white scour' with concentrated preparations of vitamin A, administered in various forms, were unsuccessful. If the vitamin was given before 'white scour' had started its absorption was indicated by a temporary rise in the level in the plasma, and by the recovery of a portion of the vitamin from the liver *post-mortem*. If dosing was commenced or continued after the development of 'white scour', however, there was no corresponding rise in the level in the plasma, and much vitamin A was excreted in the faeces. On the other hand, calves were kept alive by feeding them on colostrum followed by separated milk. Evidence was obtained that the protective value of colostrum was associated with its globulin content, since calves inoculated with precolostrum, and fed on separated milk, survived the early critical period. Antibodies, in a concentrated form, were demonstrated in precolostrum.

The possibility that deficiency of vitamin A may at a later stage be of practical importance in the rearing of bovines in this country was suggested by the constriction of the optic nerves found in steers fed upon diets consisting largely of sugar-beet pulp. Analysis of the blood and livers of some of the animals afflicted with this disease indicated that they were deficient in vitamin A. Other affected animals had adequate reserves of vitamin A when killed, but their dietary history usually suggested the possibility of deficiency during a critical period of their development.

The Nutritive Value for the Calf of Colostrum and its Fractions.

By R. Aschaffenburg, S. Bartlett, S. K. Kon, P. Terry, S. Y. Thompson and D. M. Walker (*National Institute for Research in Dairying, University of Reading*), C. Briggs and E. Cotchin. (*Research Institute of Animal Pathology, Royal Veterinary College*)

It is uncertain which is the fraction that makes colostrum so valuable to the new-born calf. We have, therefore, separated with a Sharples supercentrifuge, into a fatty and a non-fatty fraction, Ayrshire and Shorthorn colostrum collected during the first 48 hr. after calving. The fatty fraction contained c. 75 % fat and it was later found desirable to prepare a clarified fat which did not contain any non-fatty material. The fatty fractions were homogenized with reconstituted dried skim milk to a content of c. 3·5 % fat, while non-vitaminized margarine was dispersed in the non-fatty fraction to the extent of c. 2 % fat. We also made diets of untreated colostrum, of reconstituted colostrum (separated colostral fat rehomogenized with the non-fatty fraction) and of a 'synthetic' colostrum consisting of the reconstituted milk (as described below) supplemented with 52 mg. of carotene and 150,000 i.u. of vitamin A. All diets were stored at −25° in portions of 300 ml. in waxed carton containers and samples from several cows were bulked to provide 16 lb. to be fed over the first 2 days of life to Ayrshire and Shorthorn calves which had had no access to colostrum. Subsequently, the calves received a reconstituted milk mixture (1 lb. dried skim milk, 4 oz. glucose, 3 oz. non-vitaminized margarine, made up to 10 lb. with water) plus 3500 i.u. vitamin A and 700 i.u. vitamin D daily. Those calves given the 'synthetic' colostrum received also 500 mg. of vitamin C and 50 mg. of nicotinic acid daily for the first 10 days of life. Blood samples at birth and blood and liver samples at slaughter were taken for the measurement of vitamin A and carotene. The performance of the calves was assessed over 5 (later reduced to 3) weeks on general health, weight increase, body temperature and occurrence of scours. The maximum performance score was 10, graded down to 0 for a calf which died. The results for a total of 66 calves (32 Shorthorns, 34 Ayrshires) are summarized in the table.

Treatment 2 is clearly superior to treatment 1A and the good results evident from treatment 1 indicate that the essential factor in the aqueous phase is active in small concentrations. The four deaths which occurred on treatment 3 remain unexplained. The 'synthetic' colostrum was of little value to the calves. *Post-mortem* examination showed that of the 15 calves which died 13 had fatal scours. Vitamin A reserves at 5 weeks proved unrelated to the initial intake. However, in calves which died after having scoured profusely the reserves of vitamin A were low at death.

Treatment ...	1	1 A	2
	Crude fatty fraction	Clarified fatty fraction	Non-fatty fraction
Number of calves:			
Used	8	8	16
Died	1	4	1
Performance score*	62	34	81

Treatment ...	3	3 A	4
	Reconstituted colostrum	Stored untreated colostrum	'Synthetic' colostrum
Number of calves:			
Used	16	11	7
Died	4	—	5
Performance score*	68	92	20

$$* \quad \frac{\Sigma \text{ Actual scores} \times 100}{\Sigma \text{ Possible scores}}.$$

Observations on the Vitamin C Metabolism of the Piglet. By R. Braude, S. K. Kon and J. W. G. Porter. (*National Institute for Research in Dairying, University of Reading*)

Recent work by Braude, Kon & Thompson (1946) and Braude, Coates, Henry, Kon, Rowland, Thompson & Walker (1947) has shown that both sow's colostrum and milk are appreciably richer in ascorbic acid than those of most other species. It seemed of interest, therefore, to investigate whether the piglet, unlike the adult pig, is dependent on an exogenous source of vitamin C.

The vitamin C in sow's colostrum, goat's milk and in a mixture of goat's and cow's milk was destroyed by irradiation with a mercury-vapour lamp. Newly-born male piglets were removed from the sow at

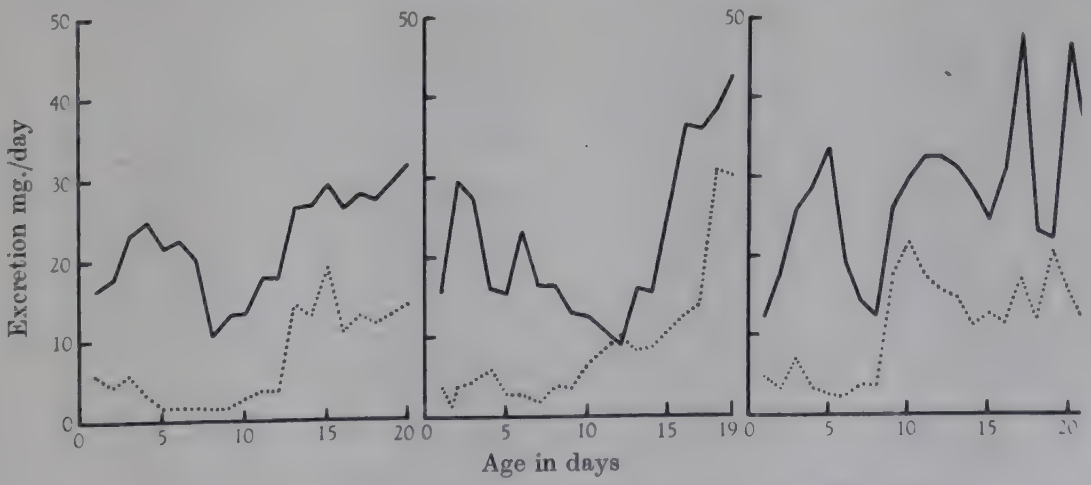

Fig. 1. Total urinary excretion of ascorbic acid (mg./day) by piglets receiving (——) or not receiving (......) this factor.

birth, kept separate in metabolism cages, and given successively these diets. Litter-mates received the same diets to which ascorbic acid had been added to make its concentration equivalent to that in sow's colostrum and milk. During the first 24 hr. the piglets were fed by hand at 2 hr. intervals; subsequently they helped themselves from self-feeders which were refilled four times during the day. The piglets on both diets grew satisfactorily. There was some indication that the control piglets utilized their food more efficiently than those on the deficient diet.

The urine of each piglet was collected at intervals and kept at 0°. Samples from 24 hr. collections were analyzed each day for ascorbic acid, using a modification of Stewart & Sharp's (1945) indophenol method. Tests showed that there was no loss of ascorbic acid during collection and storage.

The results for three pairs of piglets are shown in Fig. 1. The piglets on the vitamin C-free diet excreted small amounts of ascorbic acid during the first 8–10 days of life; a pronounced increase then occurred and the subsequent secretion, although not constant was markedly greater than that during the first period. The control piglets excreted substantial quantities during the first few days when colostrum was fed; the excretion then fell and rose again at about the same time as it increased in the deficient pigs.

This marked change in excretion at 8–10 days would suggest either that ascorbic acid synthesis does not begin until the piglet is about a week old, or that there is then a marked change in the requirement of vitamin C.

The piglets were killed after 3 weeks and samples of various body tissues were analyzed for ascorbic acid. With the exception of the adrenals, where the content was similar for all piglets, the tissues of the deficient animals contained only about half as much vitamin C as those of the controls.

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Metabolism of Phenanthrene. By E. Boyland and G. Wolf

A compound was isolated from the urine of rats injected with phenanthrene which is apparently identical with that reported by Young (1947). It is optically inactive and melts at 189–190°. From its analysis and by analogy with the metabolism of anthracene and naphthalene, it is thought to be a dihydroxydihydrophenanthrene. It gives a diacetate (m.p. 171°) and is easily dehydrated by aqueous mineral acid to a phenanthrol. This, by comparison with a synthetic specimen and through derivatives, was shown to be 9-phenanthrol. The phenanthrenediol is therefore probably 9:10-dihydroxy-9:10-dihydrophenanthrene. On oxidation with potassium permanganate it yields 9:10-phenanthraquinone and with lead tetra-acetate diphenyl-2:2′-dialdehyde. A preliminary investigation of the ultra-violet absorption spectrum of the compound, kindly carried out by Dr Roe and Mr Beale, confirms this structure, as the spectrum resembles that of 9:10-dihydrophenanthrene.

The 9:10-dihydroxy-9:10-dihydrophenanthrene prepared from phenanthrene by osmic acid oxidation according to Criegee, Marchand & Wannowius (1942), was not identical with the phenanthrenediol isolated from rat urine.

From preliminary investigations it appears that rabbits dosed with phenanthrene excrete a mixture of phenanthrene diols in the urine, as not only has 9:10-dihydroxy-9:10-dihydrophenanthrene been isolated, but also a phenanthrene-diol which yields a mixture of 1-phenanthrol and 2-phenanthrol on dehydration in ethanolic hydrochloric acid. This diol is therefore presumably 1:2-dihydroxy-1:2-dihydrophenanthrene.

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The Effect of Lipoid and Non-lipoid Solvents on the Rate of Elimination and the Carcinogenic Potency of 3:4-Benzpyrene after Subcutaneous Injection in Mice. By H. WEIL-MALHERBE. (*Department of Physiology, Medical School, University of Durham*)

It has been shown (Weil-Malherbe & Dickens, 1946) that the use of synthetic tricaprylin containing low concentrations (3 %) of cholesterol or phospholipids as a solvent for the subcutaneous injection of 3:4-benzpyrene may profoundly affect the rate of benzpyrene elimination and the incidence of induced sarcomas. The higher rate of elimination caused by the addition of cholesterol was associated with a higher tumour incidence, whereas in presence of phospholipids a slower elimination was observed together with a lengthening of the latent period of induced sarcomas. No such correlation could be obtained by ourselves or others when more complex natural oils and fats were used as solvents. A systematic study of simple synthetic or near-synthetic solvents was therefore undertaken in order to obtain confirmatory evidence and, if possible, an explanation of the mechanism of the 'solvent effect'.

Mice were subcutaneously injected with tricaprylin solutions of 3:4-benzpyrene containing one or more of a number of additional compounds. The effect of these additions on the rate of benzpyrene elimination was studied. The substances tested mainly belonged to the two classes of antioxidants or sterols.

Ascorbyl palmitate in 2 % solution was the only substance with a probable inhibitory effect, while α-tocopherol caused a marked acceleration. Amongst sterols cholesterol, cholestanol and sitosterol increased the elimination rate whereas epi-cholestanol, coprostanol, epi-coprostanol, stigmasterol, 7-dehydrocholesterol and ergosterol were inactive. This is exactly the situation found by Davis, Krahl & Clowes (1940) with regard to the ability of sterols to form association complexes with benzpyrene in surface films.

The higher rate of benzpyrene elimination caused by 3 % cholestanol is associated with a higher incidence of induced sarcomas. The delay in benzpyrene elimination in the presence of 2 % ascorbyl palmitate is only temporary and lasts for about 6–7 weeks. It is probably followed by a phase of accelerated elimination. Accordingly there was no effect of ascorbyl palmitate on tumour incidence.

The association between increased elimination rate and formation of co-ordination compounds is further strengthened by the fact that after injection of aqueous benzpyrene solutions made up with the aid of solubilizers the elimination is greatly accelerated. This is not due to the different physical conditions since benzpyrene if injected in ethereal solution or in aqueous colloidal solution is eliminated at a rate not much different from that observed with several oily solvents.

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The Metabolism of Nicotinamide and Related Compounds in Various Mammalian Species. By P. ELLINGER and M. M. ABDEL KADER. (*The Lister Institute, London, S.W.* 1)

A systematic comparative examination was carried out on nicotinamide metabolites eliminated in the urine by various mammals without and after extra dietary intake of nicotinamide (Nam), nicotinic acid (Nac) or nikethamide (Nik). Metabolites claimed to be observed by various workers are Nam, Nac, nicotinamide methochloride (NM), trigonelline (trig.) and nicotinuric acid. Investigations were performed on man, rat (2 strains), cat (young), guinea-pig, and rabbit (kept on cabbage or oats). The dietary intake (μg./day) was: man, not estimated; rat 0·212 Nam, 0·370 Nac; cat 1·025 Nam, 3·450 Nac; guinea-pig 0·879 Nam, 0·315 Nac; rabbit (cabbage) 1·600 Nam, 1·600 Nac; (oats) 0·208 Nam, 0·336 Nac. 24 hr. samples of urine were collected and examined for Nam, Nac, trig. and nicotinuric acid by a modified Bandier & Hold (1939) method and microbiologically by Barton-Wright's (1944) method and NM by a modification of Huff & Perlzweig's (1947) acetone method. Various doses of Nam, Nac or Nik were given to man subcutaneously and to the animals intraperitoneally. The main results are given in Tables 1 and 2.

In guinea-pigs and rabbits without extra dietary intake of Nam derivatives the acetone method showed some positive results, the values of which were not increased by injection of either of the compounds. The positive result was probably caused by urinary elimination of the coenzymes.

Rabbits eliminated after injection of Nik for about 16 hr. a urine which exhibited a strong purplish fluorescence in the 366 mμ mercury line. This fluorescence was caused by one, or possibly two or three hitherto unknown pigments which have been isolated and the properties of which are under examination.

Man, rat and cat amidate Nac and methylate Nam. Guinea-pig desaminates Nam but does not methylate Nac, rabbit desaminates Nam but methylates Nac. Nik is by all five species metabolized to Nam by removing the ethyl groups from the amino-N; this confirms the conclusions of Ellinger, Fraenkel & Abdel Kader (1947).

Table 1. *Spontaneous urinary elimination of metabolites*

(μg./day; mean of 8–53 experiments)

	Metabolite eliminated		
Species	Nam	Nac	NM
Man	2000 [635–4080]	810 [250–1260]	8630 [3210–12580]
Rat (albino)	75·5 [4–116]	0	103 [31–210]
Rat (hooded)	139·2 [84–165]	0	955 [450–2010]
Cat	0	0	288 [66–403]
Guinea-pig	0	0	0
Rabbit (on cabbage)	0	878 [503–1200]	0
Rabbit (on oats)	0	1187 [7010–1544]	0

Neither trigonelline nor nicotinuric acid were eliminated. Numbers in brackets are limiting values.

Table 2. *Urinary elimination of metabolites after injection of Nam, Nac, or Nik*

(% of injected; mean of 2–8 experiments)

		Metabolite eliminated			
Species	Compound injected (μg.)	Nam	Nac	NM	Trig.
Man	Nam 100	1·82	0	25·0	0
	Nac 100	2·48	0·89	21·2	0
	Nik 100	6·30	0	15·6	0
Rat (albino)	Nam 20	23·7	0	9·3	0
	Nac 20	23·8	27·0	1·8	0
	Nik 20	36·8	0	10·0	0
Rat (hooded)	Nam 20	15·0	0	29·7	0
	Nac 20	24·1	27·7	15·8	0
	Nik 20	36·8	0	33·1	0
Cat	Nam 40	34·8	0	28·5	0
	Nac 40	12·8	9·8	24·2	0
	Nik 40	18·9	0	16·1	0
Guinea-pig	Nam 200	0	24·2	0	0
	Nac 200	0	82·2	0	0
	Nik 50	19·2	40·8	0	0
Rabbit (on cabbage)	Nam 200	11·8	14·4	0	9·2
	Nac 200	0	63·4	0	33·5
	Nik 200	4·8	9·9	0	6·9
Rabbit (on oats)	Nam 200	7·5	13·0	0	6·4
	Nac 200	0	50·0	0	29·4

No nicotinuric acid was eliminated.

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A Colorimetric Method for the Estimation of Ergothioneine in Human Blood. By A. L. Latner. (*Department of Chemical Pathology, King's College, University of Durham*)

The methods available for the estimation of ergothioneine in blood are those of Hunter (1928) and Behre & Benedict (1929). The latter method, depending as it does on a molybdic acid reduction, would not appear to be sufficiently specific.

It has been shown that the colour reaction involved in the former method, although excellent for aqueous solutions, gives poor results with human blood. Three specimens of blood examined by this technique gave zero values and no recovery of added ergothioneine. The reaction seems to be adversely affected by the presence of free uric acid.

A new method has been elaborated, which depends on the fact that ergothionene after hydrolysis with $10N$ NaOH will give a red colour when treated with $NaNO_2$ and sulphanilic acid under controlled conditions. Good recoveries are obtained both with solutions of ergothioneine HCl and ultrafiltrates of plasma and whole blood previously diluted 1 : 4 with distilled water.

The applicability of the method to molybdic acid filtrates is under investigation, as it is felt that the elimination of ultrafiltration would make the technique more suitable for routine clinical purposes.

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Blood Ergothioneine Values in Normal Individuals and Thyrotoxicosis. By A. L. Latner and R. Mowbray. (*Departments of Chemical Pathology and Medicine, King's College, University of Durham*)

Since it was demonstrated that ergothioneine acts as a thyroid inhibitor (Lawson & Rimington, 1947), much interest has been aroused with regard to the possible significance of this substance in thyrotoxicosis, as a factor in both aetiology and treatment.

It became essential to define the normal range of ergothioneine values in human blood in order to demonstrate any changes that might occur in thyrotoxicosis. Unfortunately, the methods of estimation then available were not found suitable for this purpose.

Using the method described by one of us in the previous communication, it has been shown that the fasting red blood cell ergothioneine levels were lower than normal in four cases of thyrotoxicosis and low normal in two others. Further cases are being examined. The ergothioneine content of normal and abnormal human thyroid glands is also under investigation.

Ergothioneine is apparently not combined with plasma proteins when added to blood and so could be easily and rapidly excreted by the human kidney after intravenous injection. The thiouracils on the other hand are known to combine with plasma protein and are therefore protected from excretion. These facts might well explain the recent failure of Astwood & Stanley (1947) to demonstrate thyroid inhibitor activity of ergothioneine injected intravenously.

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The Chemical Nature of Aerosporin. By Tudor S. G. Jones. (*The Wellcome Chemical Research Laboratories, Langley Court, Beckenham, Kent*)

An antibiotic, aerosporin, was first described by Ainsworth, Brown & Brownlee (1947) as the product of the culture on a simple medium of a strain of *B. aerosporus* Greer. It was reported to be a basic substance, forming salts with mineral acids. It and the product of its hydrolysis with acids have since been found to give the ninhydrin reaction and to be optically active (Catch, 1947). The results of partition chromatography on paper (Consden, Gordon & Martin, 1944) suggest that the most active preparations, which when isolated by a variety of preparative methods attain an almost constant level of activity, are still not homogeneous. The intact material behaves as a basic polypeptide and exhibits the usual behaviour when chromatographed on paper, i.e. in butanol-acetic acid mixture, collidine or neutral phenol, it moves slowly and in ammoniacal phenol it moves almost as quickly as the solvent front. Electrometric titration shows two regions for the acceptance of protons having pK values of 10·5 and 5·8. Keeping in alkaline solution (pH 12, 4 hr.) alters the titration curve; there now appear three regions of pK 10·5, 7·3 and 2·35.

A number of preparations, after acid hydrolysis, have been found consistently to give three spots on the paper chromatogram, using a number of solvents. Two of these spots correspond with leucine and threonine. A single third spot of characteristic shape and colour indicates the presence of basic material, reacting with ninhydrin, and of low R_F values in neutral solvents and of high R_F values in alkaline media. Preparative chromatograms, cut to separate the two portions of the spot, and eluted with water, disproved the apparent inhomogeneity. The following amino-acids have been found to differ from this basic material: lysine, ornithine, homolysine, histidine, arginine, citrulline, β-alanine and glutamic acid.

The quantitative determination, in acid hydrolysates, of leucine and threonine on the paper chromatogram by the method of Woiwod (1948), indicated a ratio of 1 : 3.

The culture medium, after growth of the organism, and at various stages in the isolation of aerosporin, was found to contain *c.* 1 mg./ml. of alanine. This was absent from the original medium and no other amino-acid or peptide appeared to be present in this concentration either before or after growth of the organism. The amino-acid was separated from the culture medium by the use of the ion-exchange resin Zeokarb 215 (Permutit Co. Ltd.) by the method of Bendall, Partridge & Westall (1947) and was found to resist acid hydrolysis, consistent with its simple amino-acid nature.

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Colorimetric Estimation of Manganese by Means of the Folin-Ciocalteu Phenol Reagent.

By M. A. M. Abul-Fadl

DEMONSTRATIONS

A Photoelectric Flame Photometer.

By W. R. Domingo, W. Klyne and W. Weedon. (*Postgraduate Medical School of London, W.* 12)

The apparatus resembles in principle that of Barnes, Richardson, Berry & Hood (1945), and can be used for the determination of sodium and potassium in biological fluids. The solution, whose Na or K content is to be determined, is introduced as a fine spray into a controlled air-acetylene flame. The intensity of the emitted light (589 and 596 mμ lines for Na; 766 and 770 mμ lines for K) is measured by the response of a photoelectric cell placed behind suitable filters, and serves to determine the concentration of Na or K in the solution. The instrument is calibrated by a series of standard Na or K solutions, and must be repeatedly checked with these during use.

The following dilutions are convenient: plasma for Na, 1 in 100; serum for K, 1 in 10; urine for Na, 1 in 200; urine for K, 1 in 100.

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The Determination of Small Quantities of Water in Acetone.

By W. Klyne. (*Postgraduate Medical School of London, W.* 12)

Small quantities of water in acetone can be determined by measuring the temperature at which the acetone gives a homogeneous mixture with di*cyclo*hexyl ($C_6H_{11}\cdot C_6H_{11}$)—cf. Robertson (1943), determination of water in ethanol; Rising & Hicks (1926), determination of water in methanol.

The sample of acetone (1·0 ml.) and di*cyclo*hexyl (2·0 ml.) are mixed and warmed slowly; the temperature at which the cloudy mixture becomes homogeneous (clearing temperature C.T.) is noted.

% water (v/v) in acetone	C.T.	% water (v/v) in acetone	C.T.
0·0	16·0°	0·6	27·0°
0·1	18·2°	0·8	30·7°
0·2	20·1°	1·0	34·2°
0·3	21·9°	1·2	37·7°
0·4	23·6°	1·5	43·1°
0·5	25·3°	2·0	52·0°

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Bilirubin: Modified Procedure without Precipitation of Proteins. By R. V. Coxon and E. J. King

Colorimetric Estimation of Manganese by means of the Folin-Ciocalteu Phenol Reagent. By M. A. M. Abul-Fadl. (*Postgraduate Medical School of London, W.* 12)

Methods for determining Mn involve its oxidation to the heptavalent state and measurement of the resulting pink colour. Other colour reactions have been developed based on the catalytic properties of Mn (e.g. Szebelledy & Bartfai, 1936; Wiese & Johnson, 1939; Kun, 1947).

The method described here is an application of the reducing properties of $MnCO_3$ on the phosphotungstic-phosphomolybdic phenol reagent of Folin & Ciocalteu (1927). Conditions under which strict correlation exists between the extent of the reduction and the concentration of the Mn^{++} have been determined. Certain amino-acids (e.g. glycine, alanine) were found not only to improve the final blue colour and to eliminate any turbidity due to the insolubility of the $MnCO_3$, but also to increase considerably the reduction of phenol reagent by $MnCO_3$.

A 2 ml. sample containing 15–60 μg. Mn^{++} is treated with 1 ml. 7·5% (w/v) glycine. To this mixture 1 ml. dilute (1 in 3) Folin & Ciocalteu reagent is added, followed by 1 ml. 25% (w/v) Na_2CO_3 solution. The mixture is shaken and allowed to stand for 15–20 min. at room temperature for the maximum colour to develop. Suitable standards and blanks are prepared at the same time.

Al, Ba, Ca, Cr, Cu^{++}, Mg, Fe^{+++}, Sn^{+++}, Sr and Zn were without effect except in amounts sufficient to precipitate carbonate when Na_2CO_3 was added (any precipitate can be spun down without affecting the blue colour). Fe^{++}, Sn^{++}, and Cu^{+}, produce an intense blue colour with the phenol reagent. This can be easily overcome by boiling the solution with a few drops of conc. HNO_3, when the metals will thus be oxidized without affecting the Mn^{++}. Co, and to a lesser extent Ni, interferes by producing a similar blue colour, in presence of glycine, but not in its absence.

Recoveries of nearly 100% have been obtained from urines to which 12–60 μg. Mn^{++} have been added after evaporating the urine to dryness and extracting the previously ignited residue with acid. This treatment seems not to affect the Mn^{++}, since the blue-colour reaction with phenol reagent proceeds normally.

The method is somewhat more sensitive than the standard permanganate method. Its advantages lie in its simplicity, reproducibility, and in the very stable and easily measurable blue colours formed. It eliminates the difficulties of oxidation and of colour variation involved in the permanganate methods. It can be adapted for water analysis as well as for biological purposes.

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Determination of Silica and Alumina in Coal Miners' Lungs. By Inge Gernow. (Introduced by E. J. King)

An Improved Technique for Blood Iron Estimations with Undiluted Titanous Chloride from a Micrometer Burette made from a Tuberculin Syringe. By I. D. P. Wootton and E. J. King. (*Postgraduate Medical School of London, Ducane Rd., W.* 12)

This modification of the popular titanous chloride method for blood iron estimations lends itself to considerable accuracy with the simplest manipulations. Blood (5 ml.) is washed with distilled water from a pipette calibrated to contain into a 50 ml. pyrex beaker, a few drops of conc. HNO_3 are added and the beaker is capped with filter paper. The mixture is cautiously evaporated to dryness and then heated more strongly until fuming ceases. The beaker is placed overnight in a muffle furnace at 400°, the residue dissolved by warming with 5 ml. conc. HCl, and the solution diluted to 20 ml. with water. The indicator (1 ml. of 20% KCNS) is added and the contents titrated with undiluted commercial $TiCl_3$ to a colourless end-point.

The $TiCl_3$ is contained in a 'micrometer syringe burette', which consists of a horizontally mounted all-glass 1 ml. syringe, the plunger being driven by a micrometer screw (Trevan, 1925); the smallest division corresponds to a delivery of 0·0002 ml. The fused-on glass capillary outlet has a turned-up tip to minimize diffusion. It dips into the iron solution together with a tube delivering CO_2 for continuous stirring. The $TiCl_3$ in the burette, which is sufficient for 6–8 titrations, does not deteriorate over a period of an hour, and titrations can be made slowly to a stable end-point.

Titrations of the blood solutions are alternated with titrations of a standard iron solution washed from the same pipette to avoid calibration errors. The stock iron standard is an acid solution of ferric ammonium sulphate containing approx. 5 mg. Fe/ml.; it is standardized by iodimetric titration against $Na_2S_2O_3$ (Treadwell & Hall, 1930). The working iron standard is a 1 : 10 dilution of this, standardized by $TiCl_3$ titration against a solution of 99·7 % pure iron wire. The two methods of standardization have agreed within 0·15 %.

The precision of the titration procedure can be judged from a simple test. A series of eight equal volumes of a standard iron solution were titrated with a single burette-full of $TiCl_3$. The relative difference between the greatest and least titration figures was less than 0·2 %.

It has been found that the glass parts of the apparatus may be replaced without loss of accuracy by a tuberculin syringe with a fused-on capillary outlet.

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Experiments with Mineral Dusts in the Lungs of Animals. By E. J. King, C. V. Harrison and S. C. Ray

Differential Determination of Reduced and Oxidized Haemoglobin with a Photoelectric Photometer. By G. Bull and K. W. Lovel. (Introduced by E. J. King)

An Aid to Paper Partition Chromatography. By D. M. P. Phillips. (*Courtauld Institute of Biochemistry, Middlesex Hospital, W.* 1)

Two devices are demonstrated which allow the rapid measurement of the movement of a spot or band relative to the solvent front (R_F values) on paper partition chromatograms, by direct measurement. Both can be made very cheaply and quickly and have the advantage that any one component of a mixture can just as easily be chosen as standard and the other components referred to this instead of the usual solvent front standard of $R_F = 1$.

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from the author.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **264th Meeting** *of the Biochemical Society was held at the Westminster Hospital Medical School, 17 Horseferry Road, London, S.W. 1, on Friday,* 13 *February* 1948, *at* 2 *p.m., when the following papers were read:*

COMMUNICATIONS

Studies on the Liver Catalase of Normal and Cancerous Rats.

By H. WEIL-MALHERBE and R. SCHADE. (*Departments of Physiology and Pathology, Medical School, University of Durham, Newcastle-upon-Tyne*)

The reduction of catalase concentration in the liver of animals bearing a rapidly growing tumour elsewhere in the body has been extensively studied by Greenstein and his group (1941–3), but the mechanism of its causation is still obscure. There are two obvious possibilities: either a toxic principle which inhibits catalase itself or its synthesis is elaborated by the tumour or the tumour causes the withdrawal of a component essential for the synthesis or protection of liver catalase. Since Greenstein found no indication for the presence of a catalase inhibitor, various other hypotheses were tested. In view of the extensive degradation processes accompanying uncontrolled invasive growth and the increased quantities of proteoses and polypeptides present in the blood (Winzler & Burk, 1944) the effect of parenteral injection of foreign protein or protein split products on the liver catalase of normal rats was investigated. On the other hand, the exaggerated demands of a growing tumour for nitrogenous building materials suggested an inhibition of catalase synthesis owing to lack of supplies of required amino-acids. Consequently the effect on liver catalase of keeping tumour-bearing rats on diets of different protein levels was studied.

A high-protein diet (45 % casein) caused a significantly faster growth of the tumours and a smaller loss of body weight in tumour-bearing rats than a low-protein diet (8 % casein). The protein content of the liver was slightly reduced in normal rats on the low-protein diet. It was considerably lowered in all tumour-bearing rats, irrespective of the protein level of the diet.

The catalase concentration in *normal* rat liver extracts of equal protein content was not significantly changed either by different dietary protein levels or by daily intraperitoneal injections of a 10 % solution of peptone. It was however significantly raised by daily intraperitoneal injections of sheep serum.

The growth of transplanted Jensen rat sarcomas led to a fall of liver catalase concentration which in extreme cases reached 5 % of normal. The decrease of catalase concentration with the ratio of body weight/tumour weight followed an exponential course, although the individual scatter was very pronounced even in similar stages of tumour development. The protein level of the diet had no significant effect on the curve, not even in the initial stages. Serum injections in tumour-bearing rats were likewise ineffective when they were started 11 days after transplantation. When started 4 or 5 days after transplantation they were followed by regression of the tumour.

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The Constitution of Mycophenolic Acid.

By J. H. BIRKINSHAW, A. BRACKEN and E. N. MORGAN. (*Department of Biochemistry, London School of Hygiene and Tropical Medicine, London, W.C.* 1)

Clutterbuck & Raistrick (1933) in the light of considerable experimental work, for details of which the original paper should be consulted, put forward a partial structural formula for mycophenolic acid. Further work has led to a revision and amplification of this formula.

When mycophenolic acid and some of its derivatives are subjected to ozonization a smooth cleavage takes place at the double bond into an aliphatic and a benzenoid residue. Thus mycophenolic acid methyl ether $C_{18}H_{22}O_6$, containing two OMe groups, is split into levulic acid and an aldehyde $C_{13}H_{14}O_5$, retaining the two OMe groups. The carboxy group known to be present in the original molecule must therefore be attached to the side chain and not to the benzene nucleus.

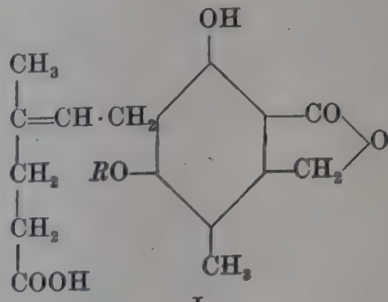
The aldehyde $C_{13}H_{14}O_5$ is converted by hypoiodite into an acid $C_{13}H_{14}O_6$, as represented by the reaction $\cdot CHO \rightarrow \cdot COOH$. On acetylation the aldehyde affords an enol acetate which undergoes further cleavage by ozone at the new double bond with production of an aldehyde $C_{12}H_{12}O_5$. These reactions indicate that the $\cdot CHO$ group is not directly attached to the benzene ring but is linked through a $\cdot CH_2\cdot$ group.

The acid $C_{13}H_{14}O_6$ can be further oxidized by alkaline $KMnO_4$ to give a tribasic acid isolated as the anhydride $C_{13}H_{12}O_7$, the relevant reaction being

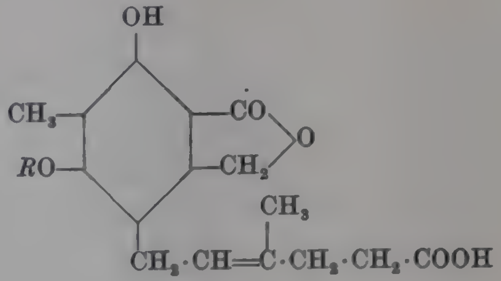
$$—CH_2\cdot O\cdot CO— \rightarrow —CO\cdot O\cdot CO—.$$

The substituents in the ring are therefore: two OMe groups *meta* to each other, $—CH_2\cdot CHO$, $—CH_2\cdot O\cdot CO—$ and $—CH_3$, all six hydrogen atoms of the ring being substituted. There are four possible structures; two can be eliminated as unlikely for various reasons, leaving I or II ($R=H$) as the probable structure for *nor*mycophenolic acid, of which I is preferred.

The position of the single methoxyl group in mycophenolic acid itself is undetermined but from the $FeCl_3$ colour reaction and the dichloroquinone chloroimide reaction, which is positive for *nor*mycophenolic and negative for mycophenolic acid it is likely that the OH is *ortho* to the potential COOH group. Hence mycophenolic acid is represented as I or II ($R=CH_3$), with preference given to I.



I



II

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Fuscin, an Antibacterial Pigment from *Oidiodendron fuscum* Robak. By S. E. Michael. (*Department of Biochemistry, London School of Hygiene and Tropical Medicine, London, W.C.* 1)

The mould *Oidiodendron fuscum* Robak when grown on Czapek-Dox medium produces an antibacterial substance. After 3 weeks' incubation at 24° the culture solution has an activity (*Staphylococcus aureus*) of 1 : 40 to 1 : 80. The active substance is readily isolated by aeration of the dark-coloured metabolism solution after filtration from the mycelium. An orange-brown powder separates which is filtered off and which on recrystallization from alcohol is obtained in the form of shining orange plates of m.p. 230°. This substance shows high antibacterial activity and is in fact the main active principle formed by the mould. The average yield is 0·3 g./l. of culture fluid. The name *fuscin* is proposed for this pigment.

In the metabolism solution fuscin is present in the colourless dihydro-form. Dihydrofuscin has actually been isolated from the solution. The oxidation to fuscin appears to be accomplished through the agency of an enzyme.

Fuscin is a compound of quinonoid character, and has the empirical formula $C_{15}H_{16}O_5$, m.p. 230°. It is soluble in organic solvents except petroleum ether, soluble in alkalis with intense purple colour. It is optically inactive; it possesses two C-Me groups, shows one active H in pyridine, none in anisole. Aldehyde reactions are negative. It gives a purplish red $FeCl_3$ reaction in alcohol. It is easily reduced by hydrosulphite or catalytically to the colourless *dihydrofuscin* $C_{15}H_{18}O_5$, m.p. 206°.

Fuscin had an appreciable inhibitory effect on 18 strains of Gram-positive bacteria with limiting dilutions varying from 1 : 80,000 to 1 : 1,280,000. It inhibited 9 Gram-negative strains at limiting dilutions varying from 1 : 5,000 to 1 : 80,000. It showed weak activity against Mycobacteria.

Fuscin reacts with thiol compounds such as cysteine and thioglycollic acid; the colourless crystalline compound formed in the latter case analyzes as fuscin hydrate. 1 thioglycollic acid.

This stable substance possesses only slightly lower antibacterial activity than fuscin itself. In the presence of higher concentration of thiol compounds, the activity is in some cases lowered, but not abolished, even with large excess (17 : 1 mol.) of thiol compound. In dilution tests zones of alternating inhibition and full activity were found, similar to the observation of Waksman & Schatz (1945) on streptomycin, and Burkholder (1944) on various antibiotics.

It is concluded that the antibacterial action of fuscin is not due to interaction with SH-groups.

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The Influence of Added Cephalin on the Thymol Turbidity and Flocculation Tests.

By N. F. Maclagan. (*Department of Chemical Pathology, Westminster Hospital Medical School, London, S.W.* 1)

Much previous work on the mechanism of this test has indicated the essential role of the native serum phospholipids in the production of the turbidity (Maclagan, 1944; Recant, Chargaff & Hanger, 1945; Kunkel & Hoagland, 1947; Maclagan & Bunn, 1947). It therefore appeared desirable to determine whether the addition of phospholipid to the reagent would improve the diagnostic value of the test and to this end sera from 45 miscellaneous cases including 12 of hepatitis in various stages were tested with and without the addition of cephalin. The cephalin was added in ethereal solution (1 % w/v), one drop being added to the 0·05 ml. of serum immediately before addition of the thymol reagent. Observations were made both on the turbidity at 30 min. and flocculation after 18 hr., using the convention recently described for recording flocculation (Maclagan, 1947). The table shows the differences between the results obtained with each test with and without cephalin addition.

Changes in thymol turbidity test produced by addition of cephalin in 45 cases (units)

Positive (12 cases)	No change	Negative (9 cases)
10, 3, 3, 2, 1, 1, 1, 1, 1, 1, 1, 1	24 cases	6, 4, 4, 4, 2, 1, 1, 1, 1

Changes in thymol flocculation test produced by addition of cephalin in 45 cases

Positive (3 cases)	No change	Negative (11 cases)
1, 1, 1	31 cases	4, 3, 2, 2, 2, 1, 1, 1, 1, 1, 1

It will be seen from this that the turbidity may be either increased or decreased by cephalin addition, but is usually unchanged. The changes were rarely such as to change a positive to a negative result or vice versa. With the flocculation test on the other hand the change if any is usually an inhibition of flocculation, which may occasionally be sufficient to reduce a strongly positive to a negative result (e.g. 4+ to 0). No particular type of case appeared to be associated with the presence or absence of this cephalin effect. It would appear therefore that the addition of cephalin is not to be recommended as a routine procedure for serum, although it is of course essential with fractions isolated electrophoretically.

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An Improved Diacetyl Reaction for the Estimation of Urea in Blood.

By V. R. Wheatley. (*Department of Chemical Pathology, Westminster Hospital Medical School, London, S.W.* 1)

The diacetyl method for blood urea of Barker (1944) has the advantage of being a direct method and in having greater sensitivity than the nesslerization-urease method. It has been found that if the condensation with diacetyl is carried out in the presence of an aromatic amine the colour produced is orange or red instead of yellow. In particular diphenylamine and its derivatives produce intense magenta colorations, that with *N*-phenylanthranilic acid being more than five times as intense as the original yellow colour with diacetyl. The reaction has been studied in detail and a method for the estimation of blood urea has been devised.

The principle of the method is as follows: the proteins are precipitated with zinc hydroxide, a portion of the filtrate, equivalent to not more than 0·02 ml. blood, is pipetted into a test tube, diacetyl monoxime and phenylanthranilic acid solutions are added followed by 18 N sulphuric acid. The tube is placed in a boiling water bath for 10 min., immediately after removal and without cooling, potassium persulphate solution is added, the tube mixed and allowed to stand at room temperature for 15 min. for the colour to develop. It is then read in a suitable colorimeter, using an Ilford spectrum green filter.

The reaction conditions have been investigated. It was found that colour production reached a steady value after 10 min. heating in the water bath. After the addition of persulphate the colour produced reaches a maximum in 15 min., and is stable for several hours. The specificity of the reaction compares favourably with the original diacetyl method except that creatinine gives a similar coloration to urea, but in this case the sensitivity is only one hundredth of that for urea. The method has been compared with the manometric method of Van Slyke & Kugel (1933). Good agreement was obtained with the majority of specimens, 80 % showing difference of less than 3 mg. urea/100 ml; in 10 % of specimens, however, the results differed by 8–10 mg./100 ml. Nevertheless, this method compares favourably with other colorimetric urea methods. It has the disadvantage that the final solution is strongly acid. Attempts to overcome this were unsuccessful since considerable dilution with water causes precipitation of the colour complex, while dilution with alcohol causes the colour to fade.

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The Use of Radioactive Isotopes in Immunological Investigations. 1. Precipitin Reactions with Ovovitellin containing P^{32}.

By G. E. Francis and A. Wormall. (*Medical College of St Bartholomew's Hospital, London, E.C.* 1)

P^{32}-containing vitellin and lipovitellin have been separated from eggs laid by hens injected with P^{32}-containing inorganic phosphate, and have been used in precipitin reactions. By the use of these labelled antigens the amount of antigen in the specific lipovitellin-antivitellin precipitates can easily be determined. In other experiments, P^{32}-containing lipid of the eggs has been used to determine the role of the egg phosphatides in the lipovitellin-antivitellin reaction. Evidence was obtained that lipovitellin-antivitellin precipitates contained a much higher ratio of phosphatide P^{32}/protein P^{32} than did the lipovitellin antigens. This preferential carrying down of phosphatide by the precipitate is specific, and it does not occur in precipitin reactions unrelated to vitellin, e.g. in the precipitation of human serum proteins by their antibodies.

The Use of Radioactive Isotopes in Immunological Investigations. 2. Fate of Injected P^{32}-containing Proteins.

By T. E. Banks, J. C. Boursnell, Hilary M. Dewey, G. E. Francis, R. Tupper and A. Wormall. (*Medical College of St Bartholomew's Hospital, London, E.C.* 1)

A preliminary study has been made of the fate of the following P^{32}-containing antigens after their intravenous injection into rabbits: phosphorylated human serum proteins, lipovitellin and vitellin. The object of these investigations was to find out which tissues take up the injected antigens, in the hope that useful information might be obtained about the site of antibody formation. The less soluble antigens, lipovitellin (which is soluble in solutions containing 5 % or more NaCl) and vitellin (which is almost insoluble in water or NaCl solutions, and which was used as a fine suspension) were used in the hope that these radioactive compounds would be specifically deposited in the reticulo-endothelial system and certain other tissues of the body.

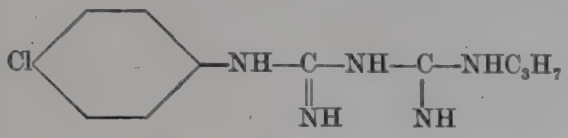
The injected phosphorylated human serum proteins rapidly disappeared from the blood stream, at least three-quarters leaving the blood during the first 3 hr. and well over 90 % during the first 24 hr. after the injection. The disappearance of the injected lipovitellin was even more rapid; only 20 % or less of the injected antigen could be detected in the blood 5–15 min. and not more than 5 % 4 or 5 hr. after the injection.

The rabbits receiving intravenous injections of the vitellin suspension died within 1 min. of the injection, presumably not because of the radioactivity of the injected antigen. Appreciable amounts of P^{32}-containing material were found in the lungs and livers of these injected rabbits, with little or none in the heart and spleen.

Some Pharmacological Properties of *p*-Chloroaniline.

By L. A. ELSON, A. SPINKS and MARY M. TOTTEY

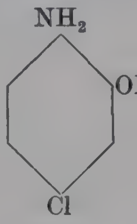
The *p*-chloroaniline molecule is a constituent of the new antimalarial drug Paludrine (I), and is readily formed from the latter by acid hydrolysis (Spinks & Tottey, 1946). It therefore seemed important to investigate the pharmacological properties of *p*-chloroaniline as a potential metabolite of the antimalarial. Its toxicity has been studied in mice and rats. The approximate acute oral LD_{50} in mice is 10 mg./20 g. When administered orally twice daily to mice over 5 days, 2 mg./20 g. resulted in the deaths of approximately 50 % of the animals. The main toxic symptoms observed were ataxia and cyanosis. During an extended chronic toxicity test in rats approximately 4 mg./rat/day were administered over a period of 6 months and the animals were then observed until the death of the last rat after 28 months. No pathological change attributable to the compound was found.



I

A method of estimating the compound was devised and studies were made of the concentrations attained by it in body fluids and tissues of mice, rats and rabbits. In some experiments simultaneous estimations of MHb concentrations were carried out. The compound attains fairly high concentrations of up to 10 mg./100 ml. in the blood and is rather uniformly distributed through the tissues, being localized to a limited extent in fatty tissues. Susceptibility to methaemoglobinaemia decreased in the order: rats; mice; rabbits. 80 % conversion of Hb to MHb was observed in rats.

Traces only of free *p*-chloroaniline could be isolated from the urine of rabbits. It appeared to be acylated in mice and rabbits, and in rabbits and rats a pronounced rise in the excretion of ethereal sulphate was detected. 5-Chloro-2-aminophenol (II) was isolated from the urine of rabbits after acid hydrolysis.



II

This metabolite accounted for only a small proportion of the administered *p*-chloroaniline, and other degradative processes must occur. Lack of knowledge of these suggests that the formation of *p*-chloroaniline from Paludrine *in vivo* would be very difficult to detect chemically. However, the high degree of methaemoglobinaemia produced by *p*-chloroaniline in experimental animals is the most pronounced of its pharmacodynamic actions, and forms a sensitive biochemical test for it. No cyanosis has been detected in man or animals after the administration of Paludrine, and it is therefore concluded that free *p*-chloroaniline cannot be formed from the latter in dangerous amount.

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Some Biochemical Properties of Methyl Bromide.

By D. R. P. MURRAY. (*Department of Zoology and Applied Entomology, Imperial College of Science and Technology, London, S.W. 7*)

Methyl bromide is extensively used in U.S.A. as a fumigant and in fire extinguishers. *In vivo* it is generally supposed to hydrolyze to methyl alcohol, which in turn is oxidized to formaldehyde, but this does not adequately explain its high toxicity. The residues of inorganic bromide in foodstuffs after fumigation have likewise been ascribed to hydrolysis, although Laug (1941) and Roehm, Schrader & Stenger (1942) found the extent of these residues varied with the nature of the foodstuff. But these facts would rather support the view that inorganic bromide arises from a reaction primarily consisting of methylation, viz. $XH + CH_3Br = XCH_3 + HBr$.

In contrast to the long-established use of alkyl halides as 'dry' alkylating reagents in heated sealed tubes, their reactivity in aqueous systems has scarcely been studied. Solutions of various substances were saturated with methyl bromide gas, or filter papers impregnated with the solutions were exposed to the gas at room temperatures. With many nitrogen and sulphur compounds but in no cases with compounds containing only C, H and O (except at extremes of pH permitting hydrolysis), bromide ions were soon detectable. Equilibrium was reached in 24–48 hr. If the filter papers were thoroughly dried no reaction occurred.

The following conditions govern the reactivity of nitrogen compounds. The nitrogen atom must have basic properties (hence amides, uric acid, etc., are inactive), but may be primary, secondary or tertiary, the three types reacting with 3, 2 and 1 molecules of methyl bromide, the latter yielding no free HBr; the final product is thus a quaternary compound. In amino-acids, polypeptides and gelatin disappearance of the amino group has been followed. The basic group must be unionized; salts including zwitterions are inactive. Hence amino-acids require addition of alkali, but peptides and proteins show a slight reactivity at pH 7. Weaker bases, including the important pyridine bases, give full reaction at neutrality or slight acidity. The methylation of nicotinic acid, which proceeds unhampered by presence of starch, etc., may have a bearing on practical fumigation.

N-methylation is apparently the only reaction taking place under these conditions. *O*-Methylation, as described for wool and methyl bromide vapour by Blackburn, Carter & Phillips (1941), has not been observed; in fact the presence of carboxyl groups is immaterial.

Dixon & Needham (1946) have shown that methyl bromide is a vesicant, but these reactions resemble those not of mustard gas, but rather of its sulphone (Boursnell, Francis & Wormall, 1946).

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Flavianates of Lysine. By J. G. HEATHCOTE. (*Department of Biochemistry, King's College, London*)

The first, and only, reference to a salt of lysine with 2:4-dinitro-1-naphthol-7-sulphonic (flavianic) acid is that of Kossel & Gross (1924). These workers claimed the preparation of a flavianate of lysine (m.p. 213°) which had N and S contents corresponding to a mono-derivative. This salt possessed only a very slight solubility in 96 % alcohol but was much more soluble in H_2O, dilute mineral acids and flavianic acid solution than the monoflavianates of histidine* and arginine. However, the salt of lysine with flavianic acid, described by Kossel & Gross, has not yet been confirmed. Carl Schmidt (1944) reports briefly that 'flavianates of lysine...have thus far not been isolated'.

By the addition of excess flavianic acid to an aqueous solution of lysine monohydrochloride, a salt has been prepared, corresponding in analysis with the diflavianate of lysine:

Calc. (%): C, 40·32; H, 3·36; N, 10·84; S, 8·28
Found (%): C, 39·97; H, 3·44; N, 10·6; S, 8·59

Lysine diflavianate is precipitated quantitatively (97 %) from aqueous solution as pale-yellow platelets which melt, when pure, at c. 275° with decomposition. Unless stoichiometric proportions are present, however, a salt with a lower melting point precipitates and recrystallization from dilute flavianic acid solution becomes necessary.

On washing the crystals of diflavianate with ethanol some change of colour occurred towards the orange shade of arginine monoflavianate, together with a lowering of melting point, suggesting the partial formation of a lower salt. Lysine monoflavianate was, in fact, prepared by dissolving the diflavianate in the minimum quantity of hot water and adding excess ethanol to the solution. The orange needles separating from the mixture at 5° melted at 213° with decomposition and had the following analysis:

Calc. (%): C, 41·74; H, 4·35; N, 12·17; S, 6·97
Found (%): C, 40·83; H, 4·58; N, 11·61; S, 6·89

It is evident, therefore, that the salts of lysine which have been prepared are, respectively, the diflavianate, hitherto undescribed, and the monoflavianate, identical with that described by Kossel & Gross.

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* Kossel's monoflavianate of histidine was later shown by Vickery (1927) to be contaminated with the diflavianate.

Excess flavianic acid has been used by some workers to free diets from arginine for nutritional work. Bunney & Rose (1928) found that all histidine was removed at the same time from a casein hydrolysate, by this procedure. It is now apparent that removal of lysine, in part at least, may also take place. The estimation of arginine by precipitation as diflavianate (Vickery, 1940) should also be reconsidered in the light of the present findings. The existence of lysine diflavianate might also explain the previous isolation of several unidentified flavianates from protein hydrolysates, e.g. by Beveridge & Lucas (1944).

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DEMONSTRATIONS

A Simplified Apparatus for the Determination of Oxygen Consumption in Small Animals.

By N. F. MACLAGAN

Estimation of Urinary Pregnanediol.

By N. F. MACLAGAN and V. R. WHEATLEY

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from the author.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **Annual General Meeting** *of the Biochemical Society was held in the Department of Biochemistry, University College, Gower Street, London, W.C. 1, on Friday, 12 March, 1948, at 2 p.m., when the following papers were read:*

COMMUNICATIONS

The Prosthetic Groups of the Intracellular Haems of *Corynebacterium diphtheriae* and their Relationship to the Cytochromes.

By W. A. Rawlinson and J. H. Hale. (*Departments of Chemical Pathology and Bacteriology, University College Hospital Medical School, London*)

The growth and metabolism of toxigenic strains of *Corynebacterium diphtheriae* are influenced by the concentration of inorganic iron salts in the culture fluid. At very low Fe levels growth is poor. The production of coproporphyrin and of toxin is maximal at 0·14 mg. Fe/ml. (Pappenheimer, 1947) and for each additional 4 atoms of Fe, 4 molecules of porphyrin and 1 of toxin fail to appear in the culture fluid but there is an increase of haem components within the cells. Pappenheimer (1947), after identifying cytochrome *b*, suggested that porphyrin and exotoxin were derived from this respiratory carrier. This hypothesis was again offered without further support in the recent paper by Pappenheimer & Hendee (1947).

We have found absorption bands in cell suspensions, reduced with sodium hyposulphite at (601), 562 and 531 mμ. and the addition of pyridine shifts the band maxima to 587, 557 and 527 mμ. These results together with other evidence confirm the findings of Fujita & Kodama (1934) and Pappenheimer & Hendee (1947), that the main haem compounds are cytochrome *b* and cytochrome oxidase.

The intracellular haems from cells grown at relatively high Fe levels have been extracted with acetone-HCl and a crude haem residue obtained free from other pigments. The development of a new process of separation, involving the phase distribution of haems between HCl-pyridine and ether, has facilitated the study of these substances. From the HCl-pyridine phase the prosthetic group of cytochrome *b* has been isolated in crystalline state and identified as protohaemin IX. Determinations have been made of extinction coefficients (as pyridine haemochromogen) of the red-green haem remaining in the ether phase, thereby providing data for the determination of the prosthetic group of cytochrome oxidase in tissue extracts.

The phase separation of haems has been applied to ox heart muscle and the pigment isolated from the ether fraction is similar to that obtained from *C. diphtheriae*. The removal of iron from these green haemins gave a rhodo type porphyrin and further work is in progress to identify its precise structure.

A positive cysteine reaction and oxime formation have indicated the presence of a labile formyl group. Marked changes in absorption spectra are observed after chemical alteration of the labile formyl group in these substances. This fact makes it pertinent to suggest that some of the confusion existing over the cytochrome *a* components, cytochrome oxidase and the respiratory ferment of Warburg might be related rather to the lability of a formyl group than to inherent differences between the protein moieties.

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The Respiratory Quotient of the Mammary Gland.

By S. J. Folley and T. H. French. (*National Institute for Research in Dairying, University of Reading*)

A number of authors have reported that the mean R.Q. of the lactating gland, determined by the method of arterio-venous sampling on the cow and goat, is greater than unity. However, apart from the uncertainties inherent in this method, the published values are not altogether decisive because of their variability, many individual results being less than 1. Some reports of experiments on perfused udders also give quotients less than 1 (for references see Kay (1947) and Folley (1948)). A series of determinations on the intact lactating cow made in this laboratory with particular care as to technique of blood sampling was reasonably consistent and gave a mean quotient of 1·25 (Bottomley & Folley, unpublished experiments).

We have now measured the R.Q. on surviving slices of rat mammary tissue by the method of Dickens & Simer (1931), with bicarbonate-Ringer containing 0·2 % glucose as substrate. A total of 26 determinations on 10 lactating rats have given a mean quotient of 1·56, range 1·30–1·86. In the absence of added substrate on the other hand, the R.Q. is below unity. Both in presence and in absence of glucose the oxygen uptake (measured in phosphate buffer by the direct method) is practically linear for 3 hr.; the Q_{O_2} (glucose) of some specimens being equal to −10.

It appears that lactose is ineffective as a substrate and that the remaining carbohydrate reserves of the tissue are very low; numerous analyses of the glycogen in fresh tissue put its concentration as low as 0·02 % in the case of the lactating rat and only slightly more in several other species.

The existence of a high R.Q. together with a fairly active respiration *in vitro*, raises interesting questions in relation to the pathways of glucose metabolism in this tissue.

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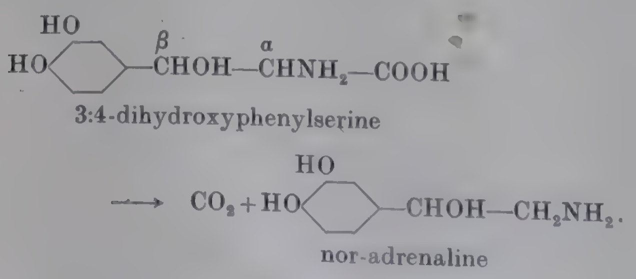
Enzymic Formation of (−)-nor-Adrenaline.

By H. Blaschko, Pamela Holton and G. H. Sloane Stanley. (*Department of Pharmacology, University of Oxford*)

β-3:4-Dihydroxyphenylserine, first prepared by Rosenmund & Dornsaft (1919), was tested as substrate of mammalian decarboxylases. It was found that extracts of the guinea-pig's kidney and suprarenal gland did not decarboxylate this amino-acid. The kidney extracts will rapidly decarboxylate L-3:4-dihydroxyphenylalanine, whereas the suprarenal extracts were found to be without dopa decarboxylase activity.

Carbon dioxide was formed when 3:4-dihydroxyphenylserine was incubated anaerobically with an acetone-dried preparation of *Streptococcus faecalis* R which contained L-tyrosine decarboxylase. The amount of CO_2 formed was equivalent to approximately one-half of the amino-acid added. This suggested that the following reaction had taken place:



$$(HO)_2C_6H_3\text{—}\overset{\beta}{C}HOH\text{—}\overset{\alpha}{C}HNH_2\text{—}COOH \longrightarrow CO_2 + (HO)_2C_6H_3\text{—}CHOH\text{—}CH_2NH_2.$$

We therefore examined the action of the incubated sample on the arterial blood pressure of the spinal cat; a high pressor activity was found. Calculation, from the amount of carbon dioxide formed, of the amine content of our solution and comparison of its pharmacological activity with that of (±)-nor-adrenaline showed that the amine formed had approximately twice the pressor activity of racemic nor-adrenaline; it must therefore have been (−)-nor-adrenaline.

Our findings make it possible to relate the configuration of 3:4-dihydroxyphenylserine to that of (−)-nor-adrenaline. The amino-acid has two asymmetric carbon atoms; four different configurations are therefore theoretically possible. We used a sample of the amino-acid prepared by Dalgliesh & Mann (1947). Dr Mann informs us that this specimen is a racemic mixture, containing only two stereoisomers. Since it is known that the bacterial enzyme is specific for L amino-acids, it follows that one-half of our specimen has the L configuration at the alpha carbon atom and the configuration of (−)-nor-adrenaline at the beta carbon atom.

The authors are grateful to Dr F. G. Mann for the sample of the amino-acid used in these experiments.

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The Amino-acids Present in Trypsin and the Trypsin Inhibitor from Soya Bean.

By ELIZABETH WORK. (*Department of Chemical Pathology, University College Hospital Medical School, London*)

Crystalline samples of trypsin and soya bean trypsin inhibitor were hydrolyzed with 6N-HCl and subjected to paper partition chromatography according to the method of Consden, Gordon & Martin (1944). The following amino-acids were identified in both substances: aspartic acid, glutamic acid, glycine, alanine, serine, threonine, valine, leucine, isoleucine, histidine, proline, tyrosine, arginine, lysine, cystine. Besides these, the inhibitor contained normal amounts of phenylalanine and methionine. The amount of these two amino-acids present in trypsin was exceptionally low, probably less than 0·5 %.

Leucine and isoleucine were easily separated and identified by a one-dimensional technique using a *tert*-amyl alcohol/water mixture in an atmosphere of diethylamine, the solvent being allowed to run over the paper for 4 days. By this method, a good separation of phenylalanine and methionine was also obtained.

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The Absorption of Vitamin A.

By EVA EDEN (*Dunn Nutritional Laboratory*) and K. C. SELLERS (*Institute of Animal Pathology, University of Cambridge*). (Introduced by T. MOORE)

Drummond, Bell & Palmer (1935), and McCoord, Breese & Baum (1943) demonstrated an increased concentration of vitamin A in the lymph collected from the thoracic duct following oral administration of the vitamin. Popper & Volk (1944) observed a fluorescence in the lacteals of the rat's gut, typical of vitamin A 25 min. after similar treatment. In the present investigation, the portal blood as well as the lymph was studied as a possible pathway of absorption of vitamin A.

Eleven yearling bullocks, weighing 150–250 kg., were dosed orally with vitamin A in the form of halibut-liver oil, emulsified in reconstituted separated milk. The dose of vitamin A was adjusted to 5000 i.u./kg. of body weight, and the animals were slaughtered at intervals varying from 2 to 24 hr. after dosing. Two undosed animals were included in the series. Vitamin A was estimated in the jugular blood before dosing and in the systemic and portal bloods collected at slaughter. At the same time lymph glands from the intestinal and other regions of the body were removed, allowed to drain, and vitamin A estimated in the lymph thus obtained.

The vitamin A content of the blood increased after dosing, but in no case was the level in the portal blood greater than that of the systemic blood. Vitamin A levels in the lymph draining the mesenteric glands, more especially those draining the duodenum, rose significantly, but not in the lymph collected from other regions of the body.

	Vitamin A (i.u./100 ml.)	
	Undosed	Dosed
Blood: Before dosing	87	72
At slaughter: Systemic	83	162
Portal	90	147
Lymph: Intestinal lymph:		
Duodenal	225	1500
Jejunal	185	1028
Ileal	130	459
Colonic	132	169
Non-intestinal lymph	130	175

Confirmatory results have been obtained in experiments performed with sheep and rats. A few samples of intestinal lymphs from sheep have been fractionated, and in these about 95 % of the vitamin A was in the ester form but small amounts of vitamin alcohol were detected.

So far we have been unable to obtain conclusive evidence that vitamin A is carried from the intestine by the portal blood. On the other hand the lymph draining the small intestine appears to be the main pathway by which the vitamin A reaches the general circulation.

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Inhibition of Rat-Liver Succinoxidase by Synthetic Oestrogens and Related Substances.

By E. M. Case and F. Dickens. (*Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W.* 1)

It was shown by McShan & Meyer (1946) that some oestrogens, particularly synthetic oestrogens of the stilboestrol series, were powerful inhibitors of the succinoxidase system of rat liver and pituitary tissues in concentrations of the order of 10^{-4}M. Some evidence was produced that certain structural modifications led to parallel diminution in both oestrogenic and inhibitory potency. If such a consistent and specific relation could be established it would have obvious importance as a potential clue to the physiological mechanism of oestrogenic action *in vivo* and might moreover throw light on the not unconnected phenomenon of carcinogenicity. However, after examination of an extended series of oestrogenic substances and compounds closely allied chemically we can find no grounds for such a generalization. Some non-oestrogenic substances inhibit strongly, while certain highly potent oestrogens, e.g. 7-methyl*bis*dehydrodoisynolic acid and β-(6-methoxy-2-naphthyl)-α, α-dimethyl-*n*-valeric acid are almost completely devoid of influence upon rat-liver succinoxidase. It is likely that the presence of phenolic hydroxyl groups in the molecule is associated with the inhibition.

McShan & Meyer considered that the inhibition which they observed was exerted upon the cytochrome oxidase component of the system and not upon succinic dehydrogenase. We found that this was by no means invariably the case for all the compounds we tested. One component or the other, or both, might be affected, in unpredictable relative degrees. However, one substance which was tried, viz. *p-p'*-dihydroxystilbene, yielded results of peculiar interest in that a concentration (2×10^{-4}M) which totally inhibited the overall oxidation of succinate was without effect upon either cytochrome oxidase or succinic dehydrogenase. The interference must therefore occur at some stage or process which links these two enzyme complexes. The existence of such an intermediate stage has been postulated on a number of occasions, from the observations of Hopkins, Lutwak-Mann & Morgan (1939) to those of Stoppani (1947) and of Ball, Anfinsen & Cooper (1947). It should be possible to employ this specific inhibitor to make some progress towards its characterization.

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Studies on the Synthesis of Ethereal Sulphates *in vivo*.

By J. C. Laidlaw and L. Young. (*Department of Biochemistry, University of Toronto*)

There has been some controversy concerning the possible utilization of administered inorganic sulphate for the synthesis of ethereal sulphates in the animal body (Rhode, 1923; Muldoon, Shiple & Sherwin, 1924; Hele, 1924). This matter has now been studied in the rat with the aid of Na_2SO_4 labelled with radioactive sulphur (S^{35}). In this work 2-naphthylamine and 2-naphthol were employed as ethereal sulphate precursors, for in earlier work in this laboratory it had been established by the isolation of the ethereal sulphates from the urine of the rat that 2-naphthylamine is converted in this animal to the ethereal sulphate of 2-amino-1-naphthol (Manson, 1947) and that 2-naphthol is converted to 2-naphthylsulphuric acid (Berenbom, 1947).

When radioactive Na_2SO_4 was given to rats by intraperitoneal injection, 5–6 % of the administered S^{35} was excreted in the urine in the following 72 hr. in the ethereal sulphate fraction and 86–90 % in the inorganic sulphate fraction. When these rats were dosed with 2-naphthylamine by stomach tube or by subcutaneous injection and at the same time were injected intraperitoneally with radioactive Na_2SO_4, it was found that in the following 72 hr. 44–48 % of the administered S^{35} was excreted in the ethereal sulphate fraction of the urine and 44–49 % in the inorganic sulphate fraction. The amounts of Na_2SO_4 injected (approx. 0·1 mg. of sulphate S per rat) were small and less than 2 % of the average daily excretion of total sulphate S in the urine. The results obtained suggested that the administered sulphate had participated in the formation of the ethereal sulphate of 2-amino-1-naphthol in the rat. This was confirmed by the isolation of the radioactive ethereal sulphate of 2-amino-1-naphthol from the urine of rats dosed by subcutaneous injection with 2-naphthylamine and injected intraperitoneally with radioactive Na_2SO_4. Furthermore, radioactive 2-naphthylsulphuric acid was isolated from the urine of rats which had been given 2-naphthol by subcutaneous injection and radioactive Na_2SO_4 by intraperitoneal injection. The specific activities of the isolated radioactive ethereal sulphates did not change significantly on repeated crystallization of the compounds. When radioactive Na_2SO_4 was added to rat urine containing the ethereal sulphate of 2-amino-1-naphthol or 2-naphthol no evidence of a transfer of S^{35} to the ethereal sulphate fraction of the urine in the following 72 hr. was obtained.

From the results of the present work it can be concluded that administered inorganic sulphate is utilized in the formation of ethereal sulphates in the rat.

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The Influence of Thyroxine and 2:4-Dinitrophenol on the Succinoxidase Activity of Rat Liver.

By H. G. WILLIAMS-ASHMAN. (*Department of Biochemistry, University College, London, W.C.* 1)

It has been reported that the succinoxidase activity of rat liver is increased on the administration of thyroid powder (Tipton, Leath, Tipton & Nixon, 1945) and also of thyroxine and thyrotropic hormone (Tipton & Nixon, 1946). The succinoxidase activity of rat liver is diminished in riboflavin deficiency and can be restored to normal levels on the subsequent administration of riboflavin (Axelrod, Swingle & Elvehjem, 1942); it has also been observed that the simultaneous administration of thiamine and vitamin B_2 complex will prevent the loss of weight which normally accompanies the administration of thyroid hormone to rats (Drill & Overman, 1941).

Thyroxine was found to increase the liver succinoxidase activity of adult male rats *in vivo*. The experiments were performed with paired feeding controls, the diet being fortified with thiamine, riboflavin, nicotinamide, pyridoxine and pantothenate. Administration of thyroxine over a period of 8 days resulted in a mean loss of weight in the experimental animals of less than 2 %; during the same period the controls suffered a mean gain in weight of less than 2 %.

The administration of large doses of 2:4-dinitrophenol in acute experiments did not result in any significant change in the succinoxidase activity of the livers of adult male albino rats.

Thyroxine, thyroglobulin and 2:4-dinitrophenol had no immediate significant influence *in vitro* on the succinoxidase activity of freshly prepared and dialyzed liver homogenates, and were also without influence on a purified preparation of succinic dehydrogenase.

It was observed that succinate affords considerable protection against the loss of succinoxidase activity which occurs on storage of liver homogenates at 4° C. The observation of Aloisi & Cavallini (1941) that thyroxine can activate *in vitro* preparations of succinoxidase which have lost activity as a result of cold storage could not be confirmed using liver homogenates.

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The Isolation and Partial Purification of 'Aerosporin'.

By J. R. CATCH and R. FRIEDMANN. (*Wellcome Chemical and Wellcome Physiological Research Laboratories, Langley Court, Beckenham, Kent*)

Crude 'Aerosporin', prepared according to Ainsworth, Brown & Brownlee (1947), may be purified by fractional precipitation as the helianthate, successive portions of methyl orange solution being added to the neutral solution of the antibiotic in water or aqueous methanol. Helianthic acid in dilute aqueous pyridine is a more convenient reagent. The aerosporin is generally (although not invariably) precipitated first, followed by impurities which appear to be, like the antibiotic itself, basic peptides. The helianthate is an amorphous brown powder, almost completely insoluble in water and most solvents. The hydrochloride or sulphate may be recovered by treatment of a methanolic suspension with the appropriate mineral acid, removal of the sparingly soluble helianthic acid, and precipitation with anhydrous acetone. Further precipitation of the amorphous reineckate, and regeneration by treatment with Ag_2SO_4 and $BaCl_2$, yields a highly active hydrochloride, of which the homogeneity is still, however, uncertain. It is a colourless, amorphous deliquescent powder, m.p. 230–235° d. after softening, freely soluble in water and in methanol; it is laevorotatory, having $[\alpha]^{20^\circ}_{5461}$ up to -42° in water. It is labile to alkali but fairly stable to acid. A positive biuret test is given and a positive ninhydrin reaction, both before and after acid hydrolysis. The Molisch, Sakaguchi, Pauly, and Hopkins-Cole reactions (for carbohydrates, monosubstituted guanidines, iminazoles, and indoles respectively) are negative. No characteristic absorption or fluorescence is shown in the ultra-violet. The elementary composition of the most active hydrochloride is C, 45·5; H, 7·7; N, 15·8; Cl, 14·0%. Sulphur and phosphorus are absent. The factor responsible for renal damage (Brownlee & Bushby, 1948) is generally still present, although some preparations contain relatively small amounts.

Many base precipitants and acid azo-dyes give precipitates with solutions of the antibiotic. The picrate and flavianate are amorphous and gummy. Treatment with benzoyl chloride or carbobenzoxychloride in aqueous sodium bicarbonate solution yields amorphous water-insoluble products, devoid of antibiotic activity.

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The Chemical Nature of 'Aerosporin'. Part II. Isolation of a New Natural Amino-Acid.

By J. R. CATCH and TUDOR S. G. JONES. (*Wellcome Chemical Research Laboratories, Langley Court, Beckenham, Kent*)

The products of the acid hydrolysis of 'Aerosporin' have been shown in Part I (Jones, 1948) to contain a basic substance, reacting with ninhydrin in solution, but not identified with any of a large variety of amino-acids. This substance is readily isolated from the hydrolysate of the most active preparations of the antibiotic as the dipicrate, m.p. 184–185°. The yield is good but the removal of the basic constituent is not complete, as is shown by chromatography on paper. Micro-analysis indicates the empirical formula $C_{16}H_{16}N_8O_{16}$. Regeneration of the picrate with HCl, and repeated recrystallization from aqueous ethanol, gives a monohydrochloride as laths or plates, m.p. 228–230° d. It is optically inactive; elementary analysis is in agreement with the formula $C_4H_{11}N_2O_2Cl$. Recrystallization in the presence of excess HCl gives a salt, m.p. 195–198° d., which approaches the dihydrochloride in composition but is less well-defined than the monohydrochloride, to which it again reverts on recrystallization in the absence of excess HCl.

A sample of synthetic DL-$\alpha\gamma$-diaminobutyric acid, prepared from DL-glutamic acid by the method of Adamson (1939), gave corresponding derivatives having the same appearances, melting points, mixed melting points and elementary compositions as those obtained from the antibiotic. Chromatographic analysis on paper of the natural and synthetic amino-acids gave, alone or in admixture, spots indistinguishable in position and colour, using several solvents. The electrometric titration curves of the two were superimposable, and indicated that the pK_A values for the three functional groups are of the same order as those of lysine and ornithine.

The most active fractions of the antibiotic have already been shown to contain leucine and threonine as components (Jones, 1948); the third, hitherto unidentified component has now been characterized as DL-$\alpha\gamma$-diaminobutyric acid. It is believed that this is the first report of the occurrence of this acid in Nature.

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The Effect of Diabetic Blood Plasma on Hexokinase *in vitro*.

By R. H. Smith. (*Department Biochemistry, University College, London, W.C.* 1.) (Introduced by F. G. Young)

De Wesselow & Griffiths (1936) observed that the blood plasma of elderly, obese, glycosuric patients, when injected into rabbits, diminished the hypoglycaemic action of insulin in a manner closely resembling that observed by other workers (e.g. Young, 1936) with anterior pituitary extracts. Similar instances of insulin antagonism by sera from insulin resistant patients have been reported by Glen & Eaton (1938), Felder (1946) and Lowell (1947).

In the light of the discovery of C. F. Cori and his co-workers (Colowick, Cori & Slein, 1947) concerning the ability of insulin to counteract the inhibition of hexokinase by anterior pituitary extract *in vitro*, it seemed possible that the anti-insulin effects observed by de Wesselow & Griffiths might be associated with the presence of a hexokinase inhibitor in the plasma.

Fractions of fresh blood plasma of a number of elderly, obese, glycosuric patients have been prepared by the alumina adsorption method and tested for hexokinase inhibiting properties following Colowick *et al.* (1947). In no instance has any significant insulin-reversible inhibition of brain hexokinase activity been obtained with these preparations.

The assistance of Prof. H. P. Himsworth and Dr M. E. Morgans in selecting the patients and obtaining the blood samples used in this work is gratefully acknowledged.

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Antagonism by Amino-acids of Renal Tubule Damaging Substances present in 'Aerosporin' Preparations.

By G. Brownlee and Eileen I. Short. (*Wellcome Physiological Research Laboratories, Langley Court, Beckenham, Kent*)

'Aerosporin' preparations may be more or less contaminated by a nephrotoxic impurity (Brownlee & Bushby, 1948). The lesions studied histologically in the rat and guinea pig appear to be restricted to the convoluted tubules. Estimates of the resultant proteinuria, in rats and dogs, have enabled semi-quantitative assays to be made, and potential antagonists to be tested. It is known that the nephrotoxic action of DL-serine is due to the unnatural isomer, D-serine (Artom, Fishman & Morehead, 1945), and the site of damage is the distal portion of the convoluted tubules (Wachstein, 1947*a*). Further, Wachstein (1947*b*) found DL-methionine and glutathionine, but not cysteine, thioglycollic acid or 2:3-dithiopropanol to protect against D-serine; DL-alanine, glycine, DL-threonine, and glycollic, butyric and pyruvic acids gave some protection.

We have confirmed the effects of these substances in rats and dogs by estimating the degree of proteinuria, and have further found that substances which antagonize the nephrotoxic action of D-serine, also antagonize the nephrotoxic activity of the impurity in the new antibiotic. Serine is absent from the toxic preparations, and the individual amino-acid components of the antibiotic (Catch & Jones, 1948) leucine, threonine and DL-$\alpha\gamma$-diaminobutyric acid are not themselves nephrotoxic.

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Fractionation of Diabetogenic Pituitary Extracts. By E. REID (Carnegie Research Fellow) and F. G. YOUNG. (*Department of Biochemistry, University College, London*)

Young (1939) described the fractionation of diabetogenic anterior pituitary extracts (ox) with ammonium sulphate. The diabetogenic and growth-promoting activities were found to associate in the 'pseudoglobulin' fraction, although in poor yield relative to crude extract. Alternative fractionation procedures are now being investigated, with the intact adult cat for the testing of diabetogenic activity (cf. Young, 1945). No appreciable diabetogenic activity is lost from alkaline saline extracts on dialysis, especially if the pH is maintained at approximately 8 during the dialysis. Good activity is present in the precipitate obtained by adjustment of the pH of the extract, thoroughly dialyzed at pH 8, to 6·0. This 'pH 6 precipitate' is quite heterogeneous, since some protein (the amount varying from one extract to another) precipitates at a higher pH and does not redissolve appreciably at pH 6·0. Diabetogenic activity is, in fact, associated with the protein which precipitates above pH 6·5, or possibly even higher (pH 6·75).

Published data for growth and lactogenic hormones suggests that these proteins tend to be soluble and insoluble, respectively, in the neighbourhood of pH 5·5, in the presence of a trace of inorganic salt. When solutions in 0·9 % NaCl of diabetogenic precipitates obtained from dialyzed extracts are acidified to pH 6·0, the activity remains in the supernatant fluid. Approximately 20–50 % of the activity of a crude extract can be recovered in such a 'saline supernatant' fraction, with a purification of approximately five-fold in terms of protein. If the pH is adjusted to 5·0 instead of 6·0, activity is divided between the precipitate and supernatant fluid.

The hexokinase-inhibiting pituitary factor is known to be adsorbed on to alumina and eluted by Na_2HPO_4 solution (Colowick, Cori & Slein, 1947). The diabetogenic factor behaves similarly, but adsorption on alumina is probably too non-specific a method to be of value in the purification of this factor.

In accordance with previous findings the diabetogenic and growth-promoting activities tend to associate; but it is likely that some factor more labile than growth hormone is implicated in the diabetogenic action of pituitary preparations on the *intact* adult dog or cat.

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The **266th Meeting** *of the Society was held in University College, Dublin, on Friday,* 9 *April* 1948, *at* 1.30 *p.m., when the following papers were read or taken as read:*

COMMUNICATIONS

Some Characteristics of the Nuclear Enzymes of Wheat. By R. GORDON BOOTH. (*Cereals Research Station, St Albans*)

The work of Peters (1945) on living animal tissues, and of Hutchinson, Greer & Thomas (1946) on living cereal grains indicated a close parallelism between the critical temperatures of damage (evidenced by lowered viability or death) in the two basic forms of living matter. Peters concludes that death is due either to irreversible inactivation of a nuclear enzyme system or to conversion of a cell membrane from its 'selective' living state to a non-selective semi-permeable type of membrane. This suggested that in the event of the first hypothesis being true, the most heat-labile enzyme systems of wheat might be found in or in close association with its cell nuclei and might be specifically concerned with the metabolism of nuclear contents.

Nucleotidases in wheat were estimated using an adenylic acid buffered substrate, the wheat extracts previously having been heat treated in the manner described by Booth (1944). Estimation of inorganic P was the measure of activity. The critical inactivation temperature of wheat nucleotidase was shown to approximate to 52·5°, this enzyme thus clearly being no more heat labile than phosphomonoesterase (cf. Booth, 1944). An attempt was made to estimate the nucleosidase activity of wheat using both adenosine and guanosine as substrates, activity being measured by assay of total reducing sugar and of free pentose. Under the conditions employed, no nucleosidase activity could be detected even in the presence of activators such as KCN and cysteine.

Polynucleotidase proved to be the most heat labile of the three enzyme systems and was measured, using both yeast and thymus nucleic acids as substrate. The former substrate was most readily attacked, and the extent of depolymerization was measured by the use of the MacFadyen (1934) reagent.

The optimal pH for this enzyme in wheat proved to be 6·0 and its critical inactivation temperature was 48°.

This low critical inactivation temperature, however, is not sufficiently low to explain lowered viability and death phenomena in heated wheat.

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The Carcinogenic Activity of *N*-Ethyl-3:4:5:6-Dibenzcarbazole. By A. H. M. Kirby. (*Research Department, Glasgow Royal Cancer Hospital*)

3:4:5:6-Dibenzcarbazole (DBC) is a very active carcinogen for mouse skin; it is very active indeed for the subcutaneous tissues and also causes hepatoma. The effect of *N*-methylation has already been reported from this Department (Kirby & Peacock, 1946); a marked reduction of activity occurred, especially in regard to the subcutaneous tissues and the liver. Lacassagne, Buu-Hoi, Royer & Zajdela (1947) have shown *N*-methyl-1:2-benzcarbazole to be no less carcinogenic for mouse skin than is 1:2-benzcarbazole itself.

N-Ethyl-3:4:5:6-dibenzcarbazole (EtDBC) has been synthesized by the general method for carbazoles (Stevens & Tucker, 1923); it forms colourless needles from ethanol, m.p. 98·5°. (Found: C, 89·6; H, 5·74; N, 4·75%. Calc. for $C_{22}H_{17}N$: C, 89·5; H, 5·75; N, 4·74%.) A 0·2% solution in acetone was painted thrice weekly on the nape of the neck, after clipping away hair, in male and female mice of the Cba strain; two subcutaneous injections, one in the right flank and the second in the left 144 days later, were made with 0·3 mg. in 0·25 ml. tricaprylin and 0·5 mg. in 0·25 ml. arachis oil respectively. Comparative tests were made in parallel in mice of the same strain using DBC from the batch used in our previous work.

The results of painting EtDBC show that papillomas and squamous carcinomas developed in 8/9 mice painted for more than 200 days, compared with an average latent period of 110 days in the DBC group. One female mouse developed an angiosarcoma 218 days after the first injection of EtDBC; all the other mice (7 males, 5 females) survived longer than this (2 males and 1 female are still alive—400 days after first injection), but no tumours at the sites of injection have been found. DBC caused sarcomas in 8/10 mice surviving 110 days after the first injection. Mice painted with EtDBC had no hyperplastic liver changes; the DBC group had hepatoma in 1/12 mice. 1/13 mice injected subcutaneously with EtDBC has developed hepatoma; DBC-injected mice had an incidence of 20%.

It may be concluded that EtDBC is a much weaker carcinogen than is DBC when painted on the skin of mice, but very much weaker than DBC when injected subcutaneously; the action of EtDBC on the liver is only slight. As stock mice were used for tests with *N*-methyl-dibenzcarbazole, strict comparison is difficult, but it seems fair to say that EtDBC has a carcinogenic power of the same order as that of the *N*-methyl homologue.

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The Metabolism of Stilboestrol and Hexoestrol in Man.

By F. H. Malpress. (*Department of Biochemistry, Queen's University, Belfast*)

Detailed quantitative studies on the excretion of synthetic oestrogens given in therapeutic doses to humans have not yet been reported, although Warren, Goulden & Robinson (1948), from observations based on the Dingemanse reaction, have concluded that the excretion of free stilboestrol in patients receiving a daily oral dose of 15 mg. could not exceed 5% of the dose given. Wilder Smith (1947), from indirect evidence, has claimed that less than 1% of the administered oestrogen is excreted in the free form, but as much as 50% as the monoglucuronide.

Determination of the urinary excretion of free and glucuronide forms after the injection or oral administration of stilboestrol and hexoestrol has now been made by a new and direct method (Malpress, 1948); preliminary results are given in Table 1.

Sulphate ester excretion is not accounted for in these results, but the evidence in the literature suggests that it is small, even in comparison with the glucuronide figure. In all cases excretion has been complete within 24 hr. of the last dose being given, and it seems probable that at these dosage levels more than 90% of the administered oestrogen undergoes radical metabolic change after intramuscular injection. The single result available after feeding stilboestrol suggests that, in its quantitative aspects at least, the metabolism of this oestrogen varies greatly with the route of its administration; further work is being done on this problem.

Table 1. *The excretion of stilboestrol and hexoestrol as free oestrogen (F) and glucuronide (G) in man, expressed as percentage total dose*

Patient	Oestrogen	5 mg. intramuscularly on 3 successive days		10 mg. intramuscularly single dose		5 mg. orally on 3 successive days	
		F	G	F	G	F	G
Mrs C.	Stilboestrol	1·3	3·2	0·6	4·0	—	—
,,	,,	1·0	8·1	—	—	—	—
,,	Hexoestrol	2·6	6·3	0·0	6·8	—	—
,,	,,	2·3	5·2	—	—	—	—
Mrs K.	Stilboestrol	3·9	5·0	—	—	—	—
Mrs T.	,,	—	—	—	—	71·4	14·4
Mr A.	,,	0·0*	7·7*	—	—	—	—

* Values slightly low owing to loss of one urine sample.

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The Solubility of Calcium Phosphate and Dental Tissues in Incubated Mixtures of Saliva and Flours of Different Extraction Rates.

By G. N. Jenkins and M. G. Forster. (*Department of Physiology, Medical School, Newcastle-upon-Tyne*)

Osborn, Noriskin & Staz (1937*a*, *b*), impressed by the low incidence of dental caries among South African Bantus, postulated the existence of a protective factor against caries in unrefined cereals and sugars. These authors incubated teeth with saliva and either crude or refined carbohydrates, and by visual inspection found that the crude materials caused decalcification in a smaller proportion of the teeth than did the refined products.

In the present work tricalcium phosphate, ground enamel, ground dentine and whole non-carious human teeth have been incubated with saliva and wheat flours of 70, 85 and 100% extraction. The rates of solution of these materials were measured by estimating the calcium concentration in the incubated medium.

It was found that the amount of calcium dissolving from the powdered materials in the presence of wholemeal flour was invariably about 60–70% of the amount dissolving with flour of 70% extraction, thus supporting the suggestion of a protective factor in unrefined cereals. The calcium dissolved in the presence of 85% extraction flour was midway between that found with the other flours.

The results with whole teeth were less convincing. In one experiment of 3 months' duration, involving 32 teeth, the calcium estimations did suggest a reduced rate of decalcification with wholemeal and

85% extraction flour. Two other experiments of shorter duration showed virtually no differences. Macroscopic examination of the teeth in all three experiments failed to show any obvious difference in the rate at which decalcified patches formed and enlarged, contrary to the findings of Osborn *et al.*

The mechanism of the reduction in solubility caused by the higher extraction flours is not yet clear. It is not caused either by differences in pH produced from the various flours by the salivary bacteria, or by differences in the amount of calcium released from the flours.

Preliminary experiments with molasses and pure sucrose suggest that the former also contains a protective factor but the results indicate that its nature differs from that of the high extraction flours.

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Accumulation of L-Glutamic Acid in Isolated Brain Tissue. By J. R. Stern (National Research Council Fellow). (*Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, The University, Sheffield* 10)

The method of Gale (1945) for the determination of L-glutamic acid has been applied to the study of the fate of L-glutamic acid added to brain slices. Under aerobic conditions $Q_{\text{glutamate}}$ (μl. L-glutamic acid disappearing from medium/mg. dry wt./hr.) was -2 to $-2 \cdot 5$ for guinea-pig brain cortex slices. The addition of glucose to the medium increased $Q_{\text{glutamate}}$ by 50–120%. Oxaloacetate, fructose, pyruvate, phosphoglycerate plus adenosinetriphosphate (ATP) or phosphoglycerate (in order of decreasing efficacy) also increased $Q_{\text{glutamate}}$. The following substances had little or no effect; lactate, citrate, α-ketoglutarate, succinate, α-glycerophosphate, ATP and ammonia.

When L-glutamic acid was added alone, or with oxaloacetate, succinate or lactate, to brain slices, more L-glutamic acid than amino-N disappeared, presumably because of transamination reactions. In the presence of glucose, fructose, pyruvate or α-ketoglutarate, disappearance of L-glutamic acid was accompanied by an approximately equivalent loss of amino-N.

The concentration of L-glutamic acid within brain slices (initially 220–290 μl./g. wet wt.) decreased progressively on aerobic incubation without added substrates. This fall was largely or wholly prevented by glucose, lactate, pyruvate or oxaloacetate, but not by L-glutamic acid. The addition of both glucose and L-glutamic acid to the medium caused an accumulation of L-glutamic acid within the slices to levels of 500–600 μl./g. Thus, provided glucose was present, brain slices were able to concentrate L-glutamic acid to levels 2–2·5 times that of freshly excised tissue. The internal concentration of L-glutamic acid attained increased as its external concentration was increased, a 20-fold concentration being effected at low external concentration (16 μl./ml.) and a five- to sixfold concentration at higher external concentration (94 μl./ml.). Under the latter conditions a maximal internal concentration was reached in about 30 min. In these experiments glucose could be replaced wholly or partly by fructose, lactate or pyruvate, but not by α-glycerophosphate, phosphoglycerate, citrate, α-ketoglutarate, succinate, oxaloacetate, ATP, or phosphoglycerate plus ATP. Thus, the disappearance of L-glutamic acid from the medium was accounted for partly by accumulation within the slice and partly by chemical change.

The accumulation of L-glutamic acid was inhibited when the oxidation of glucose was inhibited by luminal, malonate, azide or 5×10^{-4} M iodoacetate. When lactate was oxidized, 5×10^{-4} M iodoacetate caused no inhibition. Anaerobically, no accumulation of L-glutamic acid was observed.

Under conditions employed in brain experiments L-glutamic acid did not accumulate in slices of guinea-pig kidney cortex or rat liver or in teased rat testis.

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The Purity of Adenosinetriphosphate Preparations by Enzymic Degradation. By K. Bailey.

(*Biochemical Laboratory, University of Cambridge*)

The wide use of adenosinetriphosphate (ATP) in enzyme reactions, and the advent of commercial preparations, have necessitated the development of purity tests by biological methods. Three enzyme systems are employed: (*a*) myosin-adenosinetriphosphatase (ATP-ase) (see Bailey, 1942); (*b*) ATP-ase plus the myokinase of Colowick & Kalckar (1943); (*c*) yeast inorganic pyrophosphatase (Bailey & Webb, 1944). With pure ATP, the free phosphate split by (*b*) is twice that by (*a*); if adenosinediphosphate (ADP) is present, the phosphate in (*b*) is greater than twice that in (*a*). Freshly prepared ATP contains no inorganic pyrophosphate, but on storage as the Ba salt over long periods (2 years), an almost complete breakdown, mostly to adenylic acid and pyrophosphate, and partly to ADP and free phosphate, has been observed. The nucleotide is best stored in solution as the Na salt (pH 6·8) at −12°.

The enzymic analysis of commercial and laboratory ATP specimens prepared from skeletal muscle (rabbit and ox) has given the following results: (1) Using the Lohmann-Needham method (D. M. Needham, 1942), ATP is obtained pure after four precipitations of the Ba salt; first as the dibarium salt, then as the monobarium salt from ethanol-HCl (Kerr, 1941), again as the di- and finally as the monobarium salt. (2) In N-HCl (1 ml. samples immersed in boiling water) the true acid-labile P is obtained only after 10 min. and not after the customary 7 min., although this latter period does suffice for inorganic pyrophosphate. As a percentage of the acid-labile P, the P liberated from the author's ATP by (*a*) is *c.* 46 %, by (*b*) 93–96 %, by (*c*) 0–2 % (in fresh preparations). The best commercial specimens give with (*a*) 40–42 %, (*b*) 80–85 %, (*c*) 0–10 %. (3) No ADP has been detected either in laboratory or commercial ATP preparations, nor in the insoluble Ba salts obtained from skeletal muscle in various stages of rigor. On the other hand, enzymic tests show the presence of considerable amounts of ADP in cardiac muscle (S. V Perry, personal communication), thus confirming the chemical characterization of both ATP and ADP in heart muscle by Lohmann & Schuster (1935). For all enzyme tests, the decomposition of the Ba salt of ATP on an exchange resin (see Polis & Meyerhof, 1946) is recommended for the removal not only of Ba, but also of metal impurities such as Fe^{+++}.

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The Concentration of Cozymase and Total Nicotinic Acid in Cat Oxyntic Cells. By Norah M. Bradford, R. E. Davies, Edith Ellis and D. E. Hughes.

(*Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, The University, Sheffield* 10)

Maximally secreting mammalian oxyntic cells can have Q_{HCl} values greater than 1000μl./mg. dry wt./hr. and then secrete their own volume of acid juice every minute. If the secretory units in the pericanalicular zone of these cells (Crane, Davies & Longmuir, 1948) have turn-over numbers of 10^4 H^+ ions/secretory unit/min. (cf. McIlwain, 1946), there are 10^7 secretory units/oxyntic cell.

A hypothesis of HCl production (Crane *et al.*) requires that H atom transport by dehydrogenating enzymes is part of the mechanism by which H^+ ions are secreted, and hence that there are more than 10^7 such enzyme molecules/oxyntic cell. Experiments have therefore been made to determine the quantity of cozymase and of total nicotinic acid in cat oxyntic cells. Serial sections were cut with a freezing microtome (as described by Holter & Linderstrøm-Lang (1935) and Davenport (1939)) parallel to the surface of cylinders of cat gastric mucosa. The distribution of the various cells was determined by counts on sections regularly spaced through the thickness of the mucosa. The remaining sections were

Table 1. *Mean cozymase and total nicotinic acid concentrations in some cat cells and tissues*

Material	Mean conc. of cozymase (M)	Mean conc. of total nicotinic acid (M)
Oxyntic cells	3×10^{-3}	2×10^{-2}
Mucous cells	1×10^{-4}	2×10^{-4}
Muscularis mucosa	7×10^{-5}	3×10^{-4}
Other gastric cells	$1·5 \times 10^{-4}$	5×10^{-4}
Liver	$6·5 \times 10^{-4}$	$2·4 \times 10^{-3}$
Thigh muscle	$3·8 \times 10^{-4}$	$1·1 \times 10^{-3}$
Ventricle	$2·4 \times 10^{-4}$	$9·6 \times 10^{-4}$
Kidney cortex	$1·6 \times 10^{-4}$	$1·3 \times 10^{-3}$
Spleen	$3·8 \times 10^{-5}$	$5·2 \times 10^{-4}$

used separately for microbiological assay; cozymase was estimated by the method of Axelrod & Elvehjem (1939) with the modifications of McIlwain & Hughes (1948) and total nicotinic acid by the method of Barton-Wright (1946). The amounts in the various cells were calculated from a comparison of the serial counts and assays.

The results show that cat oxyntic cells contain much more of these substances than the other cells of the gastric mucosa. Moreover, the content is higher than that of the other tissues examined by these techniques (see Table 1). This extremely high concentration of a co-dehydrogenase ($1\cdot5 \times 10^9$ molecules/cell) and its relation to the number of secretory units is in accordance with the high energy output of actively secreting oxyntic cells.

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The Chemical Nature of 'Aerosporin'. III. The Optical Configuration of the Leucine and Threonine Components.

By TUDOR S. G. JONES. (*Wellcome Chemical Research Laboratories, Langley Court, Beckenham, Kent*)

Paper partition chromatograms of the products of the hydrolysis of 'Aerosporin' have been shown in Part I (Jones, 1948) to exhibit amino-acid spots in the positions corresponding to leucine and threonine. The method of Synge (1948) for the identification of D-amino-acids on paper has been applied to such chromatograms. The method consists of the application, by spraying, of a solution in 0·06 M-pyrophosphate buffer of the D-amino-acid oxidase of sheep's kidney (Negelein & Brömel, 1939), followed by incubation at 37° for $2\frac{1}{2}$ hr. in a humid atmosphere of oxygen. The paper is then dried and developed with ninhydrin in the usual manner. By matching the quantity of amino-acid present on the paper with equal amounts of the pure D- and L-amino-acid and mixtures thereof, the removal of the D-amino-acid can be followed.

It was found that the component occupying the leucine position was completely removed by the oxidase indicating that this amino-acid has the D-configuration. On the other hand, the spot due to the threonine component remained substantially undiminished in intensity in agreement with a predominantly L-configuration. A small proportion of a control spot of D-threonine remained after treatment with the oxidase. No light was thrown on the configuration of the third component, $\alpha\gamma$-diaminobutyric acid, for the D-form in this case is unattacked by the oxidase under the conditions of the experiment (cf. Handler, Bernheim & Klein, 1941, for lysine).

The co-operation of Dr R. L. M. Synge is gratefully acknowledged.

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The Determination of Amino-acids by Polarographic Reduction of their Copper Complexes.

By TUDOR S. G. JONES. (*Wellcome Chemical Research Laboratories, Langley Court, Beckenham, Kent*)

The copper phosphate reagent of Woiwod (1948) is suited to the polarographic determination of small amounts of α-amino-acids, and, in particular, those quantities which are found on paper following partition chromatography according to Consden, Gordon & Martin (1944).

For amino-acids in solution, a suitable small volume is added to the copper phosphate reagent diluted with an equal volume of 0·18 M-Na_2HPO_4 solution and, after removal of the dissolved oxygen by passage of inert gas, the polarogram is obtained. A polarogram of the diluted reagent is drawn on the same paper in the same manner. The increase in the limiting current for the region $-0\cdot7$ to $-1\cdot0$ V. is related to the amino-acid content of the solution polarographed. The relationship may be established beforehand under standard conditions, or an *ad hoc* determination under the exact conditions of temperature, capillary constants, etc., obtaining in the cell, may be made by the addition of a comparable quantity of the amino-acid being determined, dissolved in a suitably small volume.

The current yield is mainly dependent upon the capillary constants. Amino-acids differ in the current given by equimolar solutions; this is a direct consequence of difference in the diffusion constants of the copper complexes. This is shown by the mean values for the current given below, obtained in a number of experiments, for 5 μM solutions in the diluted copper phosphate reagent of the following amino-acids: glycine, 0·136 μA.; leucine, 0·112 μA.; $\alpha\gamma$-diaminobutyric acid, 0·225 μA.; lysine, 0·09 μA. The current bears a linear relationship to concentration over a threefold range of concentration, e.g. for glycine over the concentration range 80–260 μmole/l., the current was 2·90 (S.D. ± 0·02) μA./1000 μmole/l. The reduction potentials of the copper complexes do not appear to differ significantly.

The bearing of these results on the determination of amino-acids on the paper chromatogram is discussed.

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The Fate of *N*-acyl Derivatives of Ambamide and V335. By R. L. Hartles and R. T. Williams.

(*Department of Biochemistry, University of Liverpool*)

Hartles & Williams (1947) indicated that the lack of systemic antibacterial activity shown by Ambamide (Marfanil) and V 335 was due to their rapid deamination and oxidation *in vivo* to inactive metabolites. It was also suggested that the antibacterial activity of the drugs might be preserved *in vivo* if the biologically sensitive $-\mathrm{CH_2NH_2}$ group of these drugs could be protected by a group (such as an acyl group) which would be slowly removed *in vivo* releasing the active drug in the tissues. We have therefore synthesized a number of acylated derivatives of the type $R\mathrm{CONHCH_2C_6H_4SO_2NH_2}$ (Ambamide derivatives) and $R\mathrm{CONHCH_2C_6H_4SO_2CH_3}$ (V 335 derivatives) and studied their fate in the rabbit (RCO— being *formyl, acetyl, propionyl, butyryl, caproyl, caprylyl, capryl, lauryl, myristyl, palmityl* and *stearyl*).

For the V 335 derivatives the acetyl derivative was excreted unchanged in the urine; 75 % of the formyl and propionyl derivatives were excreted unchanged and 25 % deaminated. As the chain length of the acetyl group lengthened from C_4 to C_{10}, the amount of drug absorbed became progressively less, and at C_{12} and C_{14} none was absorbed and the drugs were found in the faeces. With the acyl derivatives C_4–C_{10} that part of the drug which was absorbed was completely deaminated and oxidized, no unchanged drug appearing in the urine. The compound which may be of therapeutic value is butyryl-V 335, for it appeared to be well absorbed, 95 % of the absorbed drug being excreted as *p*-methylsulphonyl-benzoic acid and only 5 % as unchanged material.

For the Ambamide derivatives, the formyl, acetyl and propionyl derivatives were excreted largely unchanged. The butyryl derivative was 33 % deaminated and oxidized and 66 % excreted unchanged; then the C_6, C_8 and C_{10} compounds became progressively less easily absorbed and finally the C_{12} and C_{14} compounds were not absorbed at all. The absorbed C_6, C_8 and C_{10} Ambamide derivatives were completely deaminated and oxidized to *p*-carboxybenzenesulphonamide.

It was to be expected that these acyl derivatives would have no antibacterial activity *in vitro*. Through Dr A. Spinks we were able to test a selection of these compounds *in vitro* against *C. welchii* and *M. tuberculosis*. Formyl-, butyryl-, caprylyl, myristyl-, and stearyl-Ambamide and formyl-, acetyl-, propionyl-, lauryl- and palmityl-V 335 were inactive against *C. welchii* and were not very encouraging against *M. tuberculosis*.

These compounds have not been tested *in vivo* under conditions where they might be expected to show activity.

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Carbonic Anhydrase as a Tool in Studying the Mechanism of Enzymic Reactions Involving H_2CO_3, CO_2 or HCO_3'. By H. A. Krebs. (*Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, The University, Sheffield* 10)

Carbonic anhydrase was previously used as a tool by Ferguson & Roughton (1934) to demonstrate the presence of carbamino-haemoglobin and by Krebs & Roughton to show that CO_2, and not HCO_3' or H_2CO_3, is the primary product of the action of urease on urea (Roughton, 1935). When carbonic anhydrase was added to the system yeast decarboxylase-pyruvate, the rate of CO_2 evolution was found to be retarded under certain conditions. This effect was studied with a manometric technique using Warburg manometers. To demonstrate an effect of carbonic anhydrase on carboxylase the enzyme must be highly active (decomposing >50 μl. pyruvate/ml./min. at 15°), pH must be near the neutral point, and a buffer, e.g. phosphate, must be present. No effects on carboxylase were found at pH 5·5. The observations are in agreement with the assumption that CO_2 is the primary form of carbonic acid arising during decarboxylation from pyruvate. Possible reasons for the discrepancy between these results and those of Conway & MacDonnell (1945) are discussed.

In work with carbonic anhydrase it is often of value to use specific inhibitors. Extending investigations of Mann & Keilin (1940) and of Davenport (1945) the inhibitory power of 25 sulphonamides was measured quantitatively. The concentrations of sulphonamides causing a 50% inhibition of carbonic anhydrase for some of the substances tested are shown in the following table, which will be discussed. The data refer to dilute solutions of carbonic anhydrase containing only negligible quantities of protein. The inhibitions were smaller in the presence of homogenized tissue.

	Concentration causing 50% inhibition (10^{-7} M)	
Sulphonamide	0°	15°
p-Toluenesulphonamide	1	2·4
Sulphanilamide	9	19
N^4-Acetylsulphanilamide	1·2	2·0
Marfanil	25	—
Thiophene-2-sulphonamide	3·0	3·8
2-Chloro-pyridine-5-sulphonamide	1·2	—
α-Naphthalene sulphonamide	1·6	2·0
Prontosil	0·06	—
Sulphamezathine	2200	4200
Sulphapyridine	510	1400
Sulphaguanidine	300	650

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A Periodic Classification in Spiral Form of the Biological Elements. By W. R. Fearon.

Tryptochrome: an Oxidation Derivative of Tryptophan. By W. R. Fearon.

The Urochrome Complex: Resolution of the Pigments Obtained by Oxidation in Presence of Zinc. By W. R. Fearon.

Concerning the Action of Yeast Carboxylase. By E. J. Conway and E. O'Malley. (*University College, Dublin*)

When yeast carboxylase acts on a high concentration of pyruvate in a space of constant volume and temperature, with adequate shaking, the following equation may be expected to apply over a short period, if direct formation of H_2CO_3 (or its ion) is occurring:

$$p=\frac{aRm}{\beta V_w}\left\{k't-\frac{k'}{\beta}\left(1-e^{-\beta t}\right)\right\}. \qquad (1)$$

If CO_2 is directly formed, the equation may be written

$$p=\frac{aRm}{\beta V_w}\left\{k't+\frac{km}{\beta}\left(1-e^{-\beta t}\right)\right\}. \qquad (2)$$

In these equations, $\beta=k'+km$; $k'=k_{H_2CO_3}([H]/K')$ and $k=k_{CO_2}$ (K' being the first adjusted ionization constant of H_2CO_3); $m=V_w s/(V_w s+V_g)$; $s=[CO_2]_w/[CO_2]_g$; V_w and V_g = the volumes of the liquid and gas phases; R = the rate of total CO_2 per minute; and a is a factor converting concentrations of gaseous CO_2 in the water phase to millimetres pressure reading p of the manometric fluid.

It follows that if carbonic anhydrase be present the ratio of the increase of pressure in its absence to the rate of pressure per minute when it is active is given by $-1/\beta$ for eq. (1), and by $+km/\beta k'$ for eq. (2), when t is relatively large (2 or 3 min. from pH 6·5 to 7·0 at 25°). Experiments at 25°, pH about 6·7, with laked blood (1 in 100) supplying carbonic anhydrase, with similar laked blood with cyanide—about N/200—in the companion flask inhibiting the carbonic anhydrase, showed an increased pressure with active carbonic anhydrase, to be expected from eq. (1). Allowance was made for the apparent cyanide effect as shown in control experiments without blood. (Some previous experiments at 37° and pH of 5·5 gave results quantitatively greater than could be expected from eq. (1).)

Further experiments carried out at 17° with phosphate buffer at a pH of 7·0, in which more diluted blood (1 in 500) was used to supply the carbonic anhydrase, and no blood or cyanide used in the companion flask, show the contrary result of a decreased pressure associated with carbonic anhydrase, and appear to agree with Prof. Krebs's findings (private communication).

The cause of the difference is being investigated.

Succinic Acid in Yeast. By T. Brady. (*University College, Dublin*)

When baker's yeast ferments glucose, in 5% unbuffered solution (1 : 0·6), it was shown that relatively large amounts of succinic acid enter the suspending fluid within 30–40 min. (Conway & O'Malley, 1943).

Further investigation has shown that the major portion of this acid is not formed directly from the glucose being fermented, but is already present in the cells. The succinic acid diffuses out from the cells so that the ratio of its concentrations within and without the cells is at or near to 0·1 (Conway & Brady, 1947).

Kleinzeller (1941) has shown that baker's yeast fermenting at a pH of 7·4 in bicarbonate medium can form succinic acid from glucose. Under the conditions used here, when the succinic acid which has passed into the suspending fluid is removed by centrifuging, the cells reform it to approximately the normal resting value.

Succinic acid or its precursor can be removed from the yeast cell by prolonged oxygenation. Short-period fermentation of yeast treated in this manner can give rise to acidity, with no release of succinic acid into the suspending fluid.

Experiments to show whether the succinic acid in the normal resting cell is present as the succinate ion or as some precursor, analytically determined as succinic acid, are in progress.

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Evidence for an Outer Metabolic Chamber in the Yeast Cell. By M. Downey and E. J. Conway. (*University College, Dublin*)

When yeast ferments glucose, succinic acid appears rapidly in the suspending fluid, but the ratio of succinic acid in the yeast cell to that outside is in the region 0·1–0·2. Similarly, when N/5-succinic acid is used as the suspending fluid (1 : 1) the acid enters rapidly to a similar ratio—0·17 in the average. So also does pyruvic acid which is steadily fermented at the same time. Over many hours succinic acid does not appear to penetrate to any greater extent than after a few minutes. A number of other acids, such as glyceric, malic, fumaric, etc., behave in a similar way to succinic and pyruvic acids, and their ratios when in N/5 strength outside range after an hour from 0·1 to 0·3 and are very little changed after several hours.

On the other hand, the group of ordinary aliphatic acids—acetic, propionic, butyric, etc.—appear to enter throughout the whole yeast cell, and so also does monoiodoacetic acid. With acetic acid the ratio, instead of being 0·18 as with succinic acid, is 1·7. As the external concentration is reduced the ratio increases, and the pH inside the resting cell can be calculated from the distribution, and is the same or close to that obtained by other methods.

If yeast is suspended (1 : 1) in solutions of varying strengths of glyceric acid, the ratio increases beyond 0·17 (but always only a small fraction for similar acetic acid levels), and assuming the initial volume of the outer chamber to correspond to 0·17 when the glyceric acid strength is high, the resting pH of the outer chamber can be roughly calculated. It would appear to be similar to that of the inner chamber, possibly somewhat lower. During fermentation there would appear to be a marked fall in the pH of this chamber as judged by the distribution ratio of small succinic acid concentrations.

Quantitative Aspects of the Storage of Vitamin A.

By A. W. Davies and T. Moore. (*Dunn Nutritional Laboratory, University of Cambridge*)

Observations on the percentage of vitamin A stored n the liver after large doses have given varying esults. Thus Baumann, Rising & Steenbock (1934) ound that only 10–20 % of the vitamin A which hey had administered to rats was stored in the liver, while as much as 47 % was found by Golding, Kon & Campion (1936) to be stored in pigs. Recently, Lemley, Brown, Bird & Emmett (1947) have reported that over the range of dosage which they nvestigated the percentage of storage increased with the magnitude of the dose up to an optimum point, but declined when very heavy doses were given.

In the present work groups of rats were kept on a diet deficient in vitamin A until growth had ceased, and were then cured with small doses of carotene or vitamin A inadequate to allow storage. Graded single doses of vitamin A were then given as halibut-liver oil, and the animals were killed 48 hr. later. Vitamin A was estimated in the livers by the $SbCl_3$ method.

In one experiment doses of 100, 200, 400 and 600 i.u. were given, and the mean percentages stored were 0, 13, 15 and 38. Other experiments always showed the same tendency, although the results at each level of dosing were not always consistent. Thus in a second experiment the mean percentages of storage with doses of 200, 600 and 1000 i.u. were 2·4, 27 and 27. In a third experiment, in which the higher doses were divided over several days, the percentage storage after 1000, 2000, 3000 and 4000 i.u. were 25, 42, 50 and 50. By giving single doses of 60,000 i.u. the mean percentage of storage was raised to about 80, and about 70 % of the ingested vitamin remained in the livers of rats kept on the basal diet for 1–4 weeks after dosing.

Doses of 240,000 i.u. were usually not entirely consumed, but three rats ingested this amount over a period of 3 days, with fatal results. The percentages of storage in the animals were only 2·5–3·8. In a rat which was given 30,000 i.u. daily for 25 days only 10 % was stored.

Three factors appear to lower the percentage of storage of vitamin A: (1) At low doses about 100 i.u. is lost, or applied to other purposes, before storage in the liver commences. The proportion which is 'lost', although not necessarily the absolute amount 'lost', decreases as the dose is raised until a toxic level is reached. (2) With toxic overdoses a high proportion of the dose may disappear, and lower stores are found than would result from giving a dose just below the toxic level. (3) With prolonged massive dosing, without obvious toxic symptoms, the percentage of vitamin stored may be low even when a high concentration is attained in the liver. This is presumably due to 'saturation' of the liver with vitamin A.

It is difficult to place an upper limit on the concentration of vitamin A which may be attained in the rat's liver by heavy dosing, but values of more than 20,000 i.u. are at least uncommon. This level is 50–100 times greater than values common in wild rats, but concentrations of the same order have been found by Rodahl & Moore (1943) in polar bears and bearded seals, and are also common in whales and certain sharks. It is to be inferred that in the human subject and domestic animals actual saturation of the liver with vitamin A presumably never occurs under ordinary conditions of nutrition.

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Vitamin A in the Intestine (Pyloric Caeca) of the Halibut.

By J. Glover and R. A. Morton. (*Biochemistry Department, University of Liverpool*)

Many fish, e.g. the halibut, store large quantities of vitamin A. The intestine of the halibut, especially the pyloric caeca, is known to be very rich in the vitamin (Lovern & Morton, 1939). This is in sharp contrast to the intestines of mammals which contain little or no vitamin A. We have recently shown that carotene is transformed into vitamin A in the small intestine of the rat (Glover, Goodwin & Morton, 1947). Its detection there is difficult as it is rapidly removed after formation. In the halibut intestine, however, vitamin A accumulates; indeed, 50 % of the lipid extracts may be vitamin A esters. It was thought that an examination of fresh halibut intestine might provide a clue to the origin of this large concentration of vitamin A and also to its mode of absorption.

The pyloric caeca of a number of halibut were dissected into a series of layers (mucosa, tunica propria, stratum compactum and stratum granulosum, and muscle) immediately the fish were caught. Each section of tissue was stored so as to preserve the vitamin A. The specimens were later examined for total, free and combined vitamin A. The results for the total vitamin indicated that the rich concentrate first appeared in the tunica propria. Over 40 % of the lipid extracts from this layer was vitamin A esters. In the mucosa, 8–40 % of the total vitamin present was in the alcohol form, whereas in the next layer, the tunica propria, the proportion of the free vitamin was more constant, 9–13 %.

The calculated percentages of free fatty acids in equilibrium with the vitamin A ester and alcohol in each layer agreed moderately well with those experimentally determined on bulked samples of corresponding tissues (Lovern & Morton, 1939). This suggests that not only does each layer contain a lipase but that in all probability the same lipase is concerned in the formation of both glyceryl and vitamin A esters.

The origin of the high concentrates of vitamin A in the halibut may be explained in the following way: During the absorption of the fatty acids and vitamin A from the lumen of the pyloric caeca, a lipase must be continually effecting alternate synthesis and hydrolysis of the lipid esters as they pass through the mucosa and tunica propria. It is possible that the free fatty acids and vitamin A alcohol in the form of water-soluble complexes separately diffuse through the cell membranes. When glyceryl esters are formed in the tunica propria their further progress is checked by their slow absorption into the 'lymph system' which is much less well developed in fish than in mammals (van Herwerden, 1908; Greene, 1913). Fatty acids, however, diffuse into this layer from the mucosa at a much more rapid rate than the esters can be removed, and are then synthesized by lipase into more esters which accumulate. However, a certain amount of free fatty acid still remains in the diffusible form because of the lipase equilibrium established. Its concentration will rise as the quantity of glyceride increases until it reaches a saturation level when the entry of further amounts of fatty acid is prevented.

If free vitamin A, either ingested preformed or produced from carotenoids, enters the tunica propria it may be esterified by the lipase utilizing some fatty acid already present there. As more vitamin A presents itself, further ester can be formed until the level of vitamin A alcohol complex in the tunica propria maintained by the lipase also reaches a saturation level and prevents further entry of free vitamin A. Theoretically this stage is reached when vitamin A ester attains a concentration of *c.* 50 % of the total lipids.

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Microdiffusion Methods. Improvements in the Determination of Carbon Monoxide and of Alcohol. By M. T. Ryan, J. Nolan and E. J. Conway.

Gray & Sandiford (1946) have used a microdiffusion procedure for determining carbon monoxide in blood, in which acid palladium chloride is reduced in the central chamber, an equivalent amount of free palladium being formed. The residual palladium after the use of aluminium sulphate to coagulate the colloidal metal, filtering, washing several times and making up to 50 ml. volume with some gum ghatti and potassium iodide, is determined spectrophotometrically. Instead of this spectrophotometric procedure, use is made of the fact that when the CO reduces the palladium salt, an equivalent amount of free HCl is formed, which can be directly titrated after removal of a portion into a small tube with a drop of bromophenol blue solution. Interference with the titration by colloidal palladium is prevented by incorporating some magnesium chloride in the absorbing fluid in the central chamber.

Many analyses of blood saturated with CO gave a standard deviation of 0·08 vol. %, and with different blood samples the results obtained (using coal gas as the source of CO) did not differ appreciably from the results obtained using the complete method of Horvath & Roughton (1942). The method should now be suitable for determining the oxygen combining capacity of blood as well as its CO content.

Alcohol. In the microdiffusion procedure of Winnick (1942) alcohol diffuses from 1·0 to 0·25 ml.

in the outer chamber into 0·5 ml. of acid dichromate solution in the central chamber, where it is oxidized, the residual dichromate being determined iodimetrically. By taking more advantage of the fact that the rate of absorption is inversely proportional to the volume of a given fraction of the alcohol introduced in the outer chamber, also that saturated potassium carbonate much increases the alcohol tension, practically full absorptions can be obtained in 1 hr. at room temperature.

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The Source of the Hydrogen Ions in the High Acid Formation by Yeast in Presence of KCl.

By E. J. Conway and T. Brady.

It was shown (Conway & O'Malley, 1942; 1943; 1944; 1946) that a high acidity can be developed in the suspending fluid when baker's yeast ferments unbuffered glucose solution in presence of KCl, the acidity arising from an interchange of K and H ions.

It was found later (Conway & Brady, 1947) that succinic acid anions and those associated with acid-labile carbon dioxide could account quantitatively for the absorbed K ions, or H ions exchanged. The inference was drawn at first that acids yielding these anions were sources of the H ions exchanged. This conclusion, however, is open to certain qualifications, though strong evidence can be provided that under certain conditions succinic acid provides directly a fraction of the hydrogen ions exchanged.

Concerning the increase in acid-labile carbon dioxide, when K exchanges for H ions, this increase is quantitatively similar to the absorbed K ions when succinic acid is very depleted by prior oxygenation. Evidence for this fraction being bicarbonate appears in its barium insolubility in presence of dimethylamine. Even if decarboxylation produced carbonic acid directly serious difficulties would arise in the quantitative interpretation, both with respect to the source of the hydrogen ions that might arise in such decarboxylation, and to the rate at which carbonic acid would be broken down. The nature and action of the increased acid-labile carbon dioxide is open to further interpretation.

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DEMONSTRATION

A Spectrum of Tests. By W. R. Fearon